THE ELUCIDATION OF THE INVOLVEMENT OF ENDONUCLEASE DNASE γ IN REDUCING DNA TRANSFECTION EFFICIENCY IN MAMMALIAN CELLS

By

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Abstract

Gene therapy is predicated upon efficient gene transfer. While viral vectors are the method of choice for transformation efficiency, the immunogenicity and safety concerns remain problematic. Non-viral vectors, on the other hand, have shown high degrees of safety and are mostly non-immunogenic in nature. However, non-viral vectors usually suffer from low levels of transformation efficiency and transgene expression. Thus, increasing transformation efficiency of non-viral vectors, in particular by calcium phosphate co-precipitation technique, is a way of generating a suitable vector for gene therapy and is the aim of this study.

It is a long known fact that different cell lines have different transfection efficiencies regardless of transfection methodology (Lin *et al.*, 1994). Using commonly available cell lines Madine-Darby Bovine Kidney (MDBK), HeLa and Human Embryonic Kidney (HEK-293), we have shown a decreasing trend of DNase activity based on a plasmid digestion assay. From densitometry studies, as much as a 40% reduction in DNase activity was observed when comparing HEK-293 (least active) to MDBK (most active). Using various biochemical assays, it was determined that DNase γ, in particular, was expressed more highly in MDBK cells than both HeLa and HEK-293. Upon cloning of the bovine DNase γ gene, we utilized the sequence information to construct antisense expressing plasmids via both traditional antisense RNA (pASDGneoM) and siRNA (psiRNA-S4, psiRNA-S11 and psiRNA-S16). For the construction of pASDGneoM, the 3' end of the DNase γ was inserted in opposite orientation under a cytomegalovirus (CMV) promoter such that the expression of RNA complementary to the DNase

γ mRNA occurred. For siRNA plasmids, the sequence was screened to yield optimal short sequences for siRNA inhibition. The silencing of bovine DNase γ led to an increase in transfection efficiency based on traditional calcium phosphate co-precipitation technique; stable clones of siRNA-producing MDBK cell lines (psiRNA-S4 B1 and psiRNA-S4 B4) both demonstrated 4-fold increases in transfection efficiency. Furthermore, serial transfection of antisense DNase γ plasmid pASDGneoM and reporter pCMV-β showed a maximum of 8-fold increase in transfection efficiency when the two separate transfections were carried out 4 hours apart (i.e. transfection of pASDGneoM, separated by four hours, then transfection of pCMV-β). Together, these results demonstrate the involvement of DNase γ in reducing transfection efficiency, at least by traditional calcium phosphate technique.

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List of Abbreviations

AAV	–	Adeno-associated virus
Ad	–	Adenovirus
ADA	–	Adenosine deaminase
ATA	–	Aurintricarboxylic acid
B cells	–	Bursa cells
BBS	–	BES buffered saline
BCA	–	Bicinchoninic acid
BES	–	N, N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
BIS acrylamide	–	N', N'-Methylene-bis-acrylamide
BSA	–	Bovine serum albumin
CAD	–	Caspase activated DNase
CaPi	–	Calcium phosphate
CAR	–	Coxsackie and adenovirus receptor
CAT	–	Chloramphenicol acetyl transferase
cDNA	–	Complementary DNA
CFTR	–	Cystic fibrosis transmembrane regulator
CHO cells	–	Chinese hamster ovary cells
CIP	–	Calf intestine alkaline phosphatase
CTL	–	Cytotoxic T-lymphocytes
Cu	–	Copper
dATP	–	Deoxyribose adenosine triphosphate
dCTP	–	Deoxyribose cytosine triphosphate
DEAE Dextran	–	Diethylaminoethyl dextran
DEPC	–	Diethylpyrocarbonate
dGTP	–	Deoxyribose guanosine triphosphate
DMF	–	Dimethylformamide
DMRIE	–	1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide
DMSO	–	Dimethylsulfoxide
DN1L3	–	DNase-I like 3
DNA	–	Deoxyribose nucleic acid
DNase	–	Deoxyribonuclease
dNTP	–	Deoxyribose nucleoside triphophate
DOGS	–	Droctadecylamidoglycylspermine
DOPC	–	Dioleoylphosphatidylcholine
DOPE	–	Dioleylphosphatidylethanolamine
DOSPER	–	1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propylamide
DOTAP	–	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	–	Dioleyloxypropyltrimethylammonium chloride

dsRNA	–	Double stranded RNA
DTIC	–	Dacarbazine
DTT	–	Dithiothreitol
dTTP	–	Deoxythymidine triphosphate
EDTA	–	Ethylenediaminetetraacetic acid
EIAV	–	Equine infectious anemia virus
EST	–	Expression sequence tag
EtOH	–	Ethanol
FGF	–	Fibroblast growth factor
FGFR	–	Fibroblast growth factor receptor
G-actin	–	Monomeric actin
GBM	–	Glioblastoma multiforme
HBS	–	HEPES buffered saline
HEK-293	–	Human embryonic kidney 293
HeLa	–	Henrietta Lacks cervical carcinoma cell line
HEPES	–	1-Piperazineethane sulfonic acid
HIV	–	Human immunodeficiency virus
HLA	–	Human leukocyte antigens
HSPG	–	Heparan sulfate proteoglycan
HSV	–	Herpes simplex virus
HSV-tk	–	Herpes simplex virus thymidine kinase
HVJ	–	Sendai virus
IC_{50}	–	Inhibitory concentration at 50%
IFN	–	Interferon
Ig	–	Immunoglobulin
IL	–	Interleukin
ITR	–	Invert terminal repeat
KCl	–	Potassium chloride
LacZ	–	β-galactosidase
LB	–	Luria broth
MDBK	–	Madine-Darby bovine kidney
MEL	–	Meurine erythroleukemia cells
$MgSO_4$	–	Magnesium sulphate
MHC	–	Major histocompatibility complex
MOPS	–	3-(N-Morpholino) propanesulfonic Acid
mRNA	–	Messenger RNA
NaCl	–	Sodium chloride
Na_2HPO_4	–	Sodium phosphate dibasic
NaH_2PO_4	–	Sodium phosphate monobasic
$(NH_4)_2SO_4$	–	Ammonium sulphate
NLS	–	Nuclear localization signal
NSCLC	–	Non-small cell lung cancer
$OD_{<wavelength>}$	–	Optical density at <wavelength>
ONPG	–	2-Nitrophenyl-bD-galactopyranoside
ORF	–	Open reading frame
PAGE	–	Polyacrylamide gel electrophoresis
PBS^-	–	Phosphate buffered saline (non-supplemented)
PCR	–	Polymerase chain reaction
PEI	–	Polyethyleneimine

PGC	–	Primordial Germ Cells
PMSF	–	Phenylmethylsulfonylfluoride
RACE	–	Rapid cloning of cDNA ends
RDA	–	Representative DNase activity
RISC	–	RNA-inducing silencing complex
RNA	–	Ribonucleic acid
RNAi	–	RNA interference
RNase	–	Ribonuclease
SCCHN	–	Small cell carcinoma of the head and neck
SCID	–	Severe combined immunodeficiency
SDS	–	Sodium dodecylsulphate
siRNA	–	Small interfering RNA
T cells	–	Thymus cells
TAE	–	Tris-acetate EDTA
TAP	–	Tobacco acid pyrophosphatase
TE buffer	–	Tris-EDTA buffer
TEMED	–	N', N', N', N'-tetramethylethylenediamine
TIL	–	Tumor infiltrating lymphocytes
TNF	–	Tumor necrosis factor
UV	–	Ultraviolet
VEGF	–	Vascular endothelial growth factor
VPC	–	Vector producing cells
VSV-g	–	Vesicular stomatitis virus G glycoprotein
Xgal	–	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter I

Introduction and Literature Review

Gene Therapy

Gene therapy can simply be defined as the use of genetic material to treat disease. Unlike pharmacological therapy, gene therapy seeks to correct the phenotypic defect on a genotypic scale, to permanently address the malfunction. More specifically, gene therapy comprises strategies employed to 1) repair the function of a mutated gene, 2) add new cellular functions to cells and 3) with the recent discovery of RNA interference (RNAi), silence an aberrant gene. Gene therapy can be further sub-divided by the cells which the therapy targets: germ line gene therapy and somatic gene therapy. For the ease of discussion and to avoid the controversial ethical considerations of germ line gene therapy, the approaches dealt within this document would strictly limit its scope to discussions of areas pertaining to somatic gene therapy. Somatic gene therapy differs from germ line gene therapy in the sense that the manipulated genetic constitution is not inherited by subsequent generations.

Conceptually, gene therapy has been most attractive for the treatment of inherited single-gene disorders, particularly those for which current therapies are unsatisfactory or nonexistent. The notion of correcting a genetic defect by replacing the missing gene function was especially attractive because of its conceptual simplicity, at least in theory. These diseases, as a result of single-gene defects, are known collectively as monogenic diseases.

Examples of monogenic diseases include cystic fibrosis, familial hypercholestremia and amyotrophic lateral sclerosis. With recent advances, however, gene therapy has expanded its horizon to potentially treat multigenic diseases and acquired multi-factorial diseases such as various heart diseases and cancers.

Initially, the notion of employing gene therapy to treat disease was extremely promising, especially due to the fact that most diseases contain some genetic component. Thus, early expectations of using genes as a possible treatment modality had been extraordinarily high. Aside from the multitude of diseases that gene therapy can apply to, it also represented a completely novel solution to many diseases that have no satisfactory treatments. This was particularly book-marked by the 4-year old Ashanthi DeSilva. In September of 1990, she became the first patient undergoing federally approved gene therapy (Blalese *et al.*, 1995). Ashanthi was born with homozygous deficiency for adenosine deaminase (ADA), which is responsible for the catabolism of toxic deoxynucleotides (Morgan *et al.*, 1987). The defect, in turn, leads to a shut down in the immune system known as severe combined immunodeficiency (SCID). Principally, SCID results in the selective accumulation of dATP in the thymocytes and peripheral blood B cells, leading to the inhibition of ribonuleotide reductase and DNA synthesis. A person stricken with this ailment suffers from chronic viral, fungal, protozoal and bacterial infections throughout his/her life. While the therapy did not yield a complete cure, it did help correct the deficiency so that Ashanti only takes small, weekly doses of the traditional Pegademase (PEG-ADA) drug (Blalese *et al.*, 1995).

The initial promise and expectations, however, did not come without sobering moments. Safety reassessment of gene therapy followed with the public death of an 18-year old volunteer, Jesse Gelsinger, with ornithine transcarbamylase deficiency during a phase I clinical trial at the University of Pennsylvania (Teicehler, 2000). It is believed that the

Jesse's death resulted from an overwhelming systemic inflammatory response to the high concentration of adenovirus that was injected directly into his hepatic veins (Dzau *et al.*, 2003). More recently in France, two patients were reported as having developed leukemia after receiving *ex-vivo* gene-corrected autologous bone marrow cells treating X-linked SCID (X-SCID), presumably by insertional mutagenesis. These incidents halted similar clinical trials scheduled to be performed in the United States. Consequently, these cases deterred the momentum that gene therapy has been gaining throughout the years and grounded the field nearly to a complete halt. Reassessment of the safety and feasibility of gene therapy soon followed alongside a very negative public image.

Today, the all-encompassing term "gene therapy" has been subdivided into "gene replacement therapy", "gene augmentation therapy", and "gene inhibition therapy". Whether the change in definition reflected the need for researchers to dissociate themselves from gene therapy's disappointing past, or whether it served to clarify the nuances of this growing field remain a topic of debate. One undeniable fact is that despite the various setbacks, the field continues to grow, with almost endless possibilities to the diseases to which it can potentially be applied. Below are a few examples.

Applications of Gene Therapy

The potential for applying gene therapy can be limitless. As long as the phenotypic defect stems from a dysfunction of genes, the corrective addition, replacement or inhibition of a gene function can, in theory, be applied to almost all instances. While the use of gene therapy has demonstrated varying levels of success in the pre-clinical/animal model studies, the transition to human clinical trials has been slow following the above public setbacks. Despite the small numbers of human clinical trials that have been largely confined to phases

I/II, the scope of gene therapy application has been very widespread. The applicable diseases are now known to include arthritis, cancer, autoimmune disorders, pulmonary diseases, kidney, cardiovascular and gastrointestinal ailments. As of July of 2004, there are 17 phase III clinical trials of gene-based therapies occurring throughout the world. Below are a few examples (http://www.wiley.co.uk/genmed/clinical/).

Suicide Gene as an Adjuvant to Surgical Resection and Radiation in Glioblastoma Multiforme

Gliomas are neoplasia of glial origins. They are the most common primary neoplasia of the brain in adults. Glioblastoma multiforme (GBM), the most malignant type of glioma, is inevitably and rapidly fatal despite aggressive therapy that typically includes surgical resection of the tumor and high-dose radiation, in conjunction with chemotherapy (Shapiro and Shapiro, 1998). The strategy to use gene therapy to augment surgical and radiation treatment was tested in a phase III clinical trial setting in 1996 by a group of scientists known as the GL1328 International Study Group.

Herpes Simplex Virus Type 1 Thymidine Kinase (HSV-tk) was produced by a replication-incompetent retroviral vector in vector-producing cells (VPCs) derived from murine fibroblasts. The pro-drug, ganciclovir, was also introduced along with the VPCs intratumorally. Since VPCs is a renewable resource for the retroviral vector, it represented a more preferable approach than multiple introduction of the vector itself due to immunological concerns. Intratumoral injection of the VPCs allows for the retroviral vector expression and integration of the HSV-tk gene into the tumor genome. HSV-tk then phosphorylates ganciclovir to form nucleotide-like precursors that block-replication of DNA

and thereby kill cells by apoptosis. Cells surrounding the transduced cells are also rendered apoptotic in a process called the *by-stander* effect, which presumably is due to cell-to-cell passage of toxic ganciclovir metabolites and other apoptotic factors (Freeman *et al.*, 1996).

Although the approach was both effective and selective in the destruction of tumor cells in earlier experimental studies, the clinical trial data published in the journal Human Gene Therapy in 2000 showed neither significant increase in progression-free period nor survival percentage over control groups, which represented conventional therapeutic modalities (Rainov, 2000).

Use of Vascular Endothelial Growth Factor to Treat Refractory Angina Pectoris

Refractory angina pectoris refers to the treatment-exhausted form of angina pectoris, which is a clinical collection of symptoms that includes chest pressure and chest pains. The primary cause of angina pectoris is myocardial ischemia, especially the lack of oxygen to the heart, which can potentially lead to myocardial infarction. In response to myocardial ischemia, collateral blood vessels usually develop to compensate for anoxia, but in patients that remain symptomatic, the collateral vessels fail to meet the need for enhanced blood flow during stress.

Christer Sylvén in Sweden is currently testing the use of gene therapy to treat refractory angina pectoris in a phase II/III clinical trial (GEMCRIS clinical trial number 0309-604). Vascular endothelial growth factor (VEGF) is introduced intramyocardially in plasmid DNA form. VEGF has been shown to stimulate neovascularization in various animal models. Furthermore, in a recent publication in *Circulation* (Henry *et al.*, 2003),

intracoronary infusions of recombinant human VEGF protein was shown to improve angina condition, exercise treadmill test and angina frequency by day 120. While the study examining the long-term efficacy of using VEGF in angina treatment continues, as the clinical trial began in January of 2002, the use of VEGF gene therapy seems to be promising.

Another phase II/III clinical trial exploring the use of human fibroblast growth factor (FGF) to stimulate neovascularization is also underway, conducted by Cindy Grines in the US. As an ongoing study, intracoronary administration of an adenoviral vector expressing FGF is being tested for the treatment of stable angina (Grines *et al.,* 2002 and Grines *et al.,* 2003).

Use of *p53* to Treat Various Forms of Cancer

In the past 20 years, investigation into the etiology of various human cancers attributed various genetic events to the formation and progression of cancers. Fundamentally, the loss of tumor suppressor genes and the activation of tumor promoter genes are both contributing factors to the tumor initiation event. As cancers developed, the accumulation of further genetic mutations results in a collection of genetic abnormalities that is unique for each cancer type. However, the loss of tumor suppressor genes has been unequivocally shown to lead to the formation of cancers. This was supported by the fact that germline mutations of *p53*, a known tumor suppressor gene, were associated with the rare inherited cancers classified under the Li-Fraumeni syndrome (Malkin *et al.*, 1990).

The phosphoprotein *p53* is a transcription factor that is involved in numerous cellular events associated with cell cycle control and apoptosis. It has the ability to suppress progression through the G1/S transition, thereby allowing time for DNA repair to occur. Moreover, when DNA damage reaches a "threshold" amount, *p53* also has the ability to

induce apoptosis, which is a cascade of cellular events leading to the programmed suicide of the affected cell. These two functions of *p53* combine to play a paramount role in the prevention of tumors from forming and progressing (White, 1996). Armed with this information, the restoration of aberrant *p53* function by introducing *p53* through gene therapy remains the most prevalent gene therapy approach in the phase II/III clinical trials.

Currently, John T. Hamm of the University of Louisville and John Nemunaitis of PRN Research Incorporated are independently investigating the efficacy of gene therapy on squamous cell carcinoma of the head and neck (SCCHN) with an adenoviral vector expressing wildtype *p53* by intratumoral administration (vector name: RPR/INGN 201). The phase III clinical trial involves patients with recurrent/refractory SCCHN, which is usually fatal within a year. Initial results have been promising as early phase II clinical trials saw 26 percent of patients experiencing tumor growth control and/or regression with *p53* gene therapy. The use of the adenoviral vector RPR/INGN 201 demonstrated usefulness outside of SCCHN as Stephen Swisher of the University of Texas is also testing the efficacy of the vector on advanced unresectable non-small cell lung cancer (NSCLC). While the global phase III clinical trials of these studies are still underway, the results seem to be encouraging (GEMCRIS clinical trial numbers 9709-214, 9912-366 and 0009-412).

In March of 2004, Peng Zhaohui, of SiBiono Gene Technology Company Limited, received approval for the first gene therapy medicine in the world from China's State Food and Drug Administration (SFDA). The recombinant adenoviral vector expressing *p53* will be later registered as Gendicine, as a therapeutic agent against SSCHN.

Immunomodulation as a Cancer Gene Therapy Approach

The restoration of tumor suppressor genes is not the only approach currently being tested for cancer treatment. Through the use of immuno-stimulatory agents, a biotechnology firm, Vical Incorporated, is examining the combination of a traditional chemotherapeutic agent, dacarbazine (DTIC), with a gene therapy immuno-therapeutic, Allovectin-7, on patients with metastatic or recurrent melanoma (GEMCRIS clinical trials protocol number 9802-234).

Cutaneous melanoma is one of the most prevalent forms of cancer in the U.S. While early detection and surgical excision of melanoma give a favorable prognosis, once tumor spreads beyond the skin, it is one of the most deadly forms of cancer. There are currently no effective therapies for advanced disease, and 10-year survival rates are very low.

Allovectin-7 is a gene therapeutic agent encoding the gene for the highly immunogenic major histocompatibility complex (MHC) class I transplantation antigen HLA-B7. The approach materialized when patients treated with DTIC combined with immunostimulants interferon α (IFN-α) and interleukin 2 (IL-2) resulted in clinical response as high as 60-70% of test subjects, whereas chemotherapy alone yielded responses in only 15-25% of test subjects (GEMCRIS clinical trials protocol number VCL-1005-301 Version 1.00). The plasmid, VCL-1005, encodes the HLA-B7 heavy chain and β2 microglobulin proteins. The β2 microglobulin allows for the synthesis and expression of the complete MHC complex on the cell surface to occur. The plasmid is complexed with the cationic lipid mixture DMRIE/DOPE (Figure 4) and is administered intratumorally. This study is markedly different from previous examples since it involves the use of a non-viral vector DMRIE/DOPE. As of January of 2004, the phase III clinical trial is still in progress.

The realization of the potential for economic gains has prompted a multitude of biotechnology and pharmaceutical firms to engage in active research in gene-based medicines. These pharmaceutical giants-supported companies include Vical Inc., Genetic Therapy Inc., Genovo Inc., Somatix Therapy Corp., Chiron Corp., and Sequana Therapeutics.

Gene Therapy Approaches

As discussed above, genetic methods are being developed to treat angina, vascular diseases, and cancer. The latter include methods that cause cancer cell suicide through the induction of apoptosis or necrosis, or the induction of an immune attack upon the tumor cells. While the potential of using gene-based medicine to treat diseases is widely recognized, the most optimal route for gene delivery remains a contentious topic for researchers. Aside from access to the site of administration, numerous difficulties are associated with the gene delivery process.

The delivery of a transgene to remediate a phenotypic defect can occur in at least one of two ways: 1) direct vector-mediated gene transfer or 2) indirect gene replacement with allogenic cells modified *ex-vivo* to express new genes. While each approach has its own advantages and shortcomings, all approaches nonetheless aim to correct a disease by mediating the expression of a missing protein. Direct vector mediated gene transfer approaches are generally preferable over *ex-vivo* approaches mainly because *ex-vivo* therapies are notoriously laborious (see below). However, direct vector mediated gene transfer suffers from immunological safety concerns (in the case of viral vectors) or inefficient transgene delivery and expression (for non-viral vectors).

Yet, the corrective addition of gene function(s) does not represent the sole approach viable for gene therapy. In some cases, silencing aberrant genes that are upregulated in a

disease state, such as tumor promoters, or foreign genes that are expressed to propagate bacterial, viral or fungal infections constitute alternative applications for gene based remedies. Historically, gene silencing consisted of homologous recombination aimed at permanently disrupting the gene of interest, broadly named as "gene targeting". While conceptually elegant, homologous recombination was never made its way out of the *in-vitro* setting as a means to silence disease-causing genes due to several major technical difficulties. In the early 1990s, antisense RNA was discovered as a suppression mechanism in petunias and nematodes. For a better part of ten years, antisense RNA became a hotbed of research as it had the potential to produce gene silencing in ways that were not possible with traditional gene targeting. However, antisense RNA never made a significant impact in terms of gene-based medicines primarily due to problems such as ineffective gene inactivation, non-specific gene suppression and most surprisingly, the ability of the sense RNA to mediate gene suppression. While research on antisense RNA ultimately did not translate into the clinical setting, it made several important contributions to the fundamental understanding of the underlying RNA interference (RNAi) mechanism, paving the way for the discovery of siRNA in 2001. Using variations of siRNA to mediate gene silencing, gene targeting is once again one of the hottest fields of research in gene therapy. RNAi has been linked to the experimental treatment of human immunodeficiency virus (HIV) infection, respiratory syncytial virus infection and some cancers in several pre-clinical studies with varying levels of success (http://www.wiley.co.uk/genetherapy/clinical/). Looking forward, the promise of RNAi technology is surely to become more prevalent in the clinical setting.

In-Vivo Gene Therapy

In-vivo gene therapy involves the direct vector-mediated introduction of gene functions into experimental subjects. Introduction of genetic material can be via systemic circulation, such as DNA vaccination and immunomodulation; or it may be tissue specific, as in the case of cystic fibrosis, whereby a localized gene delivery is required. Moreover, it may also be site-specific, as in the intratumoral injection of suicide genes for the treatment of some forms of cancer.

One of the difficulties involved with *in-vivo* gene therapy is the choice of vector; the considerations needed for the vector choice are often predicated upon the conditions of gene delivery. For example, adenoviral vectors are preferred for the delivery of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene to treat cystic fibrosis because of the innate tissue specificity of adenoviruses and their high transduction efficiency. However, no single gene transfer vector currently exists that encompasses all of the required characteristics needed to be a "universal" vector. For instance, while adenoviral vectors are highly efficient in the delivery of the transgene, they are also very immunoreactive, resulting in their rapid elimination from systemic circulation over subsequent re-administration. This makes recurring treatments impossible. Moreover, large doses of adenoviral vectors can lead to a large-scale inflammatory response that can endanger a patient's health.

On the other hand, the relatively non-immunogenic nature of most non-viral vectors makes them an attractive alternative. However, the notoriously low transduction efficiency and toxicity associated with some formulations hamper their development as a treatment modality. Thus, choosing an appropriate vector based on its application remains a major shortcoming associated with the *in-vivo* gene therapy approach.

Ex-Vivo Gene Therapy

Ex-vivo gene therapy, in contrast to *in-vivo* approaches, is the indirect genetic modification with autologous cells, performed outside of the patient, to express the desired transgene. The first step involves the removal of a patient's own cells. Through genetic modification, clonal expansion and selection of desired cell lines, these transgenic cells are re-introduced into the patient, via a variety of means. *Ex-vivo* gene therapy, while much more laborious and time consuming, exerts fewer criteria on the vector choice. Since transfected cells are selected for transgene expression by selectable markers, a wider range of gene transfer methods, including less efficient means such as calcium phosphate or Lipofectamine, can be employed. Moreover, the requisite genetic modifications occur outside of a patient's body. Thus, provided that the necessary purification processes occur prior to re-introduction, pro-inflammatory responses elicited by viral vectors can be sidestepped. An example of *ex-vivo* gene therapy is the expression of recombinant adenosine deaminase in lymphoid progenitor cells to treat severe combined immunodeficiency (Morgan *et al.*, 1987).

However, *ex-vivo* gene therapy is not without its own set of difficulties. Firstly, *ex-vivo* genetic modification is a multi-stage, time-consuming process, which in essence requires every treatment to be tailor-made to each patient. Aside from the labor intensive nature, *ex-vivo* gene therapy requires a patient's cells to be accessible and robust enough to survive the various stages of manipulation such as transfection/transduction, selection, clonal expansion, purification and re-introduction into the patient. Thus, large-scale development of *ex-vivo* gene therapies to treat prevalent diseases can be extremely cost-prohibitive.

siRNA Gene Silencing

In 2001, Elbashir *et al.* (2001) demonstrated the use of small interfering RNA (siRNA) in inhibiting luciferase activity in mammalian cells. However, the news did not come as a surprise to many as researchers have been using this technology in *Caenorhabditis elegans* and *Drosophila melanogaster* for many years. What was surprising was the fact that Elbashir discovered the criteria to bypass the double stranded RNA-mediated interferon response by limiting the length of the double stranded region of the interfering RNA to 21 nucleotides (Elbashir *et al.*, 2001). The potent inhibition that resulted, led to a new direction of research on RNA interference. The inhibition elicited by siRNA was not only potent but also specific, and it gave new light to years of antisense RNA research that often gave dubious results (Branch, 1998).

The ability to silence genes not only gave researchers a new avenue to study gene functions, but it also gave clinicians a new way to treat disease. Aberrant genes can cause many genetic diseases. For many infectious diseases requiring viral/fungal early gene expression, RNAi was the answer. Currently, RNA interference is being investigated to treat leukemia, neuroblastoma, carcinoma, malaria, HIV, rotavirus, hepatitis, influenza, human papilloma virus and fragile X syndrome (Cheng *et al.*, 2003).

The mechanism for RNA interference (RNAi) is conceptually simple. RNA strand containing sequence that is complementary to the target RNA forms Watson and Crick base-pair interactions with the target mRNA, leading to the formation of double-stranded RNA (dsRNA). In the cell, long dsRNAs are cleaved into short 21-25 nucleotide siRNA, by a ribonuclease known as Dicer. The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC), unwinding in the process. Activated RISC then binds to complementary transcript by base pairing interactions between the siRNA

antisense strand and the mRNA. The bound mRNA is cleaved and sequence specific degradation of mRNA results in gene silencing (McManus and Sharp, 2002).

Targeting Gene Therapy

Successful gene therapy requires the targeted delivery of the therapeutic, as localized gene delivery remains one of the difficulties plaguing gene therapy today. While some vectors can deliver the transgene with high efficiency, controlling tissue specificity is still problematic. Delivery vectors, especially viral vectors, require a specific cell surface receptor for attachment to occur. For example, adenoviral vectors bind specifically to an adhesional membrane receptor, the coxsackievirus and adenovirus receptor (CAR), which may or may not be expressed on the cells of interest. To remedy this problem, Dutch researchers C. H. Bangma and J. Trapman are investigating transductional targeting where bispecific conjugates between the monoclonal antibody against the adenoviral knob on the one side, and a molecule targeting a surface antigen on the other side are employed (Bangma, 2000). In essence, they have constructed a molecular adaptor for the adenoviral vector to bind tissue types foreign to the native adenovirus.

An alternative approach to enhance the specificity of the delivery vector is to control transgene expression with the help of a tissue specific promoter. Transcriptional targeting exploits the promoter regions of genes that are switched on only in certain tissues. While the vector may not be specific during the delivery process, transgene expression will not occur, theoretically, in the absence of the tissue specific transcriptional factor. Some of these tissue specific promoters include the prostate-specific antigen, ovarian-specific promoter and trypsinase (Robson and Hirst, 2003).

Challenges of Gene Therapy

Today, there remain numerous challenges for gene therapy before it becomes an applicable treatment modality in the US. Aside from potential treatments to transition from current phase III clinical trials to FDA approval, other technical difficulties with gene therapy vectors such as extracellular/intracellular barriers, control of transgene expression, vector choice and safety concerns remain to be resolved.

Extracellular barriers include both physical and immunological barriers. For example, epithelial and endothelial cells have the potential to block DNA transfer. Delivery methods, to this date, remain far from perfect. While systemic administration is the delivery method of choice, some cells are inaccessible by this route, such as cells of the central nervous system protected by the blood-brain barrier (Misra *et al.*, 2003). Injection in the site of action requires precise administration and usually user intervention.

Immunological barriers to gene transfer comprise both the humoral and cell-mediated immune responses. Viral vectors are especially susceptible to immune response due to the presence of viral proteins. This makes repeated dosing of viral vector-based gene therapy nearly impossible as the secondary immune response quickly eliminates additional viral vector from systemic circulation (Schagen *et al.*, 2004). Moreover, cytotoxic T-lymphocytes (CTL) can mount an immune response against the transgene, destroying cells that have been transduced and thus negating previous efforts (Muruve *et al.*, 2004). The need for a non-immunogenic and efficient vector remains a crucial aspect to the success of gene therapy in the future.

To complicate matters further, the successful delivery of the therapeutic transgene does not necessarily translate to the expression of such transgene. When exogenous DNA is transfected into a cell, it must first contend with the plasma membrane. Depending on the

vector used, receptor mediated endocytosis occurs and internalization of DNA takes place. Once internalized, DNA must escape endosomal/lysosomal degradation, which presents a major limiting step to efficient gene transfer (Ciftci and Levy, 2001). Of the small fraction of DNA that escapes into the cytosol, cytosolic endonucleases are present to digest the remaining DNA. The final barrier is the nuclear membrane, which safeguards the cell from transcribing exogenous DNA. The extremely small proportion that overcomes all of the above barriers gains access to the nucleus, where transcription occurs. The multiple barriers during transfection/transduction present numerous hurdles for gene transfer to elicit a therapeutic effect, making this process extremely inefficient (Bally *et al.*, 1999).

As stated previously, control of transgene expression presents another major limiting step that requires attention. While transductional and transcriptional targeting is currently being refined, tissue specificity during gene transfer remains an issue to be contended. Tissue specific promoters have inherent specificity, but transgene expression levels usually lag far behind those of constitutive promoters (Robson and Hirst, 2003). Thus, the need for both efficiency and specificity in a single vector remain to be satisfied. Based on the current state of gene-transfer technology, the overall effectiveness of gene therapy may rely on matching the vector to the needs of a particular disease.

Safety concern is potentially the most significant hurdle that gene therapy faces, especially with the use of viral vectors. With the publicized death of Jesse Gelsinger, the Food and Drug Administration will place further scrutiny towards the safety of these treatments. Aside from the pro-inflammatory reactions elicited by viral proteins from viral vector mediated gene transfer, numerous viral vectors allow for the integration of transgenes into the host genome. While this ensures stable and prolonged transgene expression, insertional mutagenesis may lead to activation of oncogene expression, or inactivation of tumor suppressor genes, leading to the development of cancer. This possibility was

punctuated by two patients developing leukemia during their gene therapy for the treatment of X-linked SCID (Hacein-Bey-Abina *et al.*, 2003).

While the direction of gene therapy to generate disease treatment modalities remains hopeful, there are numerous hurdles that remain. With the completion of the human genome project, the possible targets for gene modulation have grown exponentially, and the stake for realizing this technology has never been higher. As the development of gene transfer tools continues, the use of genes to treat diseases as a widespread treatment option remains to be seen.

Future of Gene Therapy

Scores of companies are researching and developing gene therapeutics in the United States and Europe. Additionally, many biotechnology and pharmaceutical companies are developing gene therapy-related products. Many of these products are already in phase I or II clinical trials (http://www.wiley.co.uk/genetherapy/clinical/). While more basic research is still required in order to understand the biology and immunology of viruses being engineered as gene therapy vectors, as well as the development of highly efficient non-viral vectors, there are vector systems that have demonstrated success in clinical trials. With the intense efforts dedicated to this field, more vector systems are sure to be developed within the next one to two years, and it appears that the clinical application of gene therapy can only expand in the years to come.

Tools of Gene Transfer

The success of gene therapy is predicated upon successful gene transfer. While other factors such as tissue specificity and expression control also warrant consideration, the need for high transduction/transfection efficiency is, by far, the most important criterion. After all, target cells that do not receive the transgene warrant little concern about expression control.

In essence, gene transfer involves the delivery of the therapeutic transgene into the target cell(s) and/or organ(s). The transgene not only carries sequences encoding the therapeutic protein, but also sequences controlling their expression. While naked DNA has demonstrated successful gene transfer when introduced into target tissue via intramuscular injection (Wahren, 1996), most clinical applications require the use of the DNA combined with a transportation vehicle known as a vector. The vector packages the genetic material and delivers it, with a degree of specificity depending on the vector, to the target cell via interaction with the target cell membrane. Once internalization takes place, the genetic material traverses numerous barriers, eventually arriving at the nucleus, where expression of the transgene occurs. Depending on the type of vector used, integration of transgene into the host genome may occur.

Vectors can be broadly separated into two categories: viral vectors and non-viral vectors. Viral vectors are recombinant viruses with portions of the viral genome replaced with the therapeutic transgene. Non-viral vectors, on the other hand, include almost all forms of DNA-packaging techniques that facilitate cellular uptake and expression of exogenous DNA. These vectors are discussed in greater detail below.

Viral Vectors

Viruses are natural gene delivery systems. Perfected by millions of years of evolution, viruses have evolved ways to efficiently deliver its genetic material, cause viral gene expression, and "hijack" host cell machinery for viral propagation (Whittaker et al., 1998). It therefore seemed logical to exploit viruses to engineer gene delivery systems for therapeutic purposes.

The viral envelope and coat proteins interact with specific cellular receptors, allowing for viral attachment, fusion and internalization (Knipe *et al.,* 1995). Since these interactions are specific, viruses show preference, or viral tropism, to certain tissues. For example, HIV selectively attaches and infects $CD4^+$ T-lymphocytes primarily. Viruses, once attached, will deliver its genetic cargo into the host cell. Together with other viral proteins, transcription of viral genes occurs with the utmost efficiency. Viral transcription, under the right circumstances, can begin as early as 7.5 minutes post transfection (Groner *et al.,* 1983).

Recombinant viral vectors exploit certain sequences known as packaging signal sequences. A packaging signal sequence adopts a certain conformation once it is recognized specifically by one of the structural proteins during the assembly of the viral core. By appending packaging sequences to the therapeutic transgene, fully infectious viral vectors are generated instead of viral progeny, resulting in an efficient delivery vector.

Although virtually all viruses can be exploited as viral vectors, the most widely used viral vectors derive from the following viruses: (1) retroviruses and other lentiviruses, (2) adenovirus, (3) adeno-associated virus and (4) Herpes simplex virus (Ratko *et al.,* 2003). Since each viral vector has distinct characteristics, such as viral tropism, ability for integration and duration of expression, no universal vector currently exists, and the vector is usually chosen based on its application (Baum *et al.*, 2003).

Table 1: Characteristics of the most commonly used gene transfer vectors

	Vector Capacity for DNA-carrying/kb	*Duration of expression*	*Inflammatory response*	*In-vivo gene delivery efficiency*
Retrovirus	*<7.5*	*Stable*	*Low*	*Low*
Adenovirus	*<8*	*Transient*	*High*	*Moderate/ Effective*
Adeno-associated virus	*<5*	*Stable*	*Low*	*Low*
Liposome	*Unlimited*	*Transient*	*Low*	*Low*
Molecular conjugate	*<10*	*Transient*	*Low*	*Low*

For a comprehensive list of gene transfer vectors, refer to Ratko *et al.*, 2003.

Retrovirus

Retroviral and lentiviral vectors are derived from type C retroviruses. These vectors can include murine leukemia virus, human immunodeficiency virus and feline immuno-deficiency virus. The viral particles are spherical, 80 to 100nm in diameter, comprising an icosahedral protein core that contains 2 copies of the 7- to 11-kb single-stranded RNA viral genome plus 3 essential enzymes: reverse transcriptase, protease and integrase (White and Fenner, 1994). The core is surrounded by a lipid envelope that carries the viral envelope glycoproteins responsible for virus attachment and entry. After attachment, the virus envelope fuses with the cell membrane, and the core moves toward the nucleus. The viral RNA is reverse transcribed to double-stranded DNA and transported into the nucleus where the viral enzyme integrase directs its insertion into the host chromosomal DNA at a semi-random site. Viral genes are transcribed from the pro-viral DNA (White and Fenner, 1994).

These recombinant retroviral vectors have been engineered with portions of the *gag*, *pol* and *env* viral sequences deleted, which render the viruses replication-defective and decrease their immunogenicity (Kim *et al.,* 1998). Because viral genes that are necessary for viral replication, encapsulation, infection, and reverse transcription are removed from the virus, a "packaging cell line" and helper plasmids are required to provide these critical functions *in trans* in order to produce the recombinant vector (Anson, 2004). The packaging cell line churns out recombinant virus carrying a therapeutic gene in place of the deleted viral genes. The therapeutic gene is carried in a viral particle that remains replication-deficient, but is still able to infect its target cell. While vector particles are immunogenic, vector-transduced cells express no viral gene products and are therefore non-immunogenic. The expression of transgene usually peaks within 72 hours and then gradually declines over weeks, months, or years because of methylation, acetylation, provirus deletion, or death of the target cell (Pannell and Ellis, 2001).

Retroviral vectors can accommodate up to 7.5kb of transgene for delivery (Verma and Somia, 1997). In their natural life cycle, retroviruses are non-lytic, which allows the stable expression of transgene occurs through the life of the transduced cell. Theoretically, the target cell genome is permanently modified, which would be an advantage when treating hereditary and chronic diseases.

One of the prerequisites for a successful transfection procedure using retroviral vectors is that the target cell must proliferate (Miller *et al.*, 1990), and the retrovirus is, therefore, not a suitable vector for *in-vivo* transduction into cells that have silent cell turnover, such as pulmonary and renal cells. Whereas murine leukemia virus derived retroviral vectors require cell division for integration to occur, lentiviral vectors may present a solution as they have the ability to integrate DNA into quiescent cells (Ratko *et al.*, 2003). Another disadvantage of retroviral vectors is that the integration of transgene occurs in a

random fashion, which is a cause of concern as insertional mutagenesis may cause the activation of oncogenes or disruption of tumor suppressor genes (Baum *et al.*, 2003). Furthermore, it is difficult to prepare high titers of viral stocks, and the expression of the transgene is difficult to control.

Retroviruses have been used mostly in *ex-vivo* gene transfer trials (Crystal, 1995). Thus far, *ex-vivo* retrovirus-mediated gene transfer in humans have demonstrated success in T cells, tumor infiltrating lymphocytes (TIL), stem cells from blood and bone marrow, tumor cell lines derived from solid tumors (Rosenberg *et al.,* 1993), hematopoietic cell lines (Medin *et al.,* 1996) and synovial cells (Jorgensen *et al.,* 1997). Moreover, therapeutic genes have been transduced employing this approach into fibroblasts (Moullier *et al.,* 1993), T cells in ADA deficiency and HIV infection, cord blood cells, placental cells, tumor cells (Oldfield *et al.,* 1993) and hepatocytes in familial hyper-cholesterolemia. The marker gene or mRNA expression after retroviral gene transfer has been observed for variable lengths of time, with the expression of transgene detected at 36 months post-transduction (Lutzko *et al.*, 1999).

Adenovirus

Adenoviral vectors are non-enveloped viruses with an 80- to 110-nm-diameter icosahedral protein shell that encases a single copy of its linear, double-stranded DNA genome of 36-38kbp in size (White and Fenner, 1994). The fiber proteins that are primarily responsible for attachment appear on 12 vertices of the icosahedron, interacting with the coxsackievirus and adenovirus receptor (CAR) (St George, 2003). Secondary attachment occurs through the penton base of the viral capsid with cell surface integrin receptors. After endocytosis, partially uncoated virus travels to the nuclear pore complex and delivers viral DNA into the nucleus, following a pH-dependent disruption of the endosome. Upon entering

the nucleus, Early gene products (E1) drive the expression of other viral genes, including E2, E3, E4 and Late genes. The E2 region encodes proteins involved in viral replication such as DNA binding protein, DNA polymerase, and terminal protein precursor. E3 proteins are responsible for reducing the antiviral immune response and block tumor necrosis factor (TNF) induced lysis or apoptosis of infected cells. E4 gene products serve a variety of functions including control of viral transcription, DNA replication and shut-off of host macromolecular synthesis and correct synthesis of unit length viral chromosome. Late genes encode viral structural proteins including capsid, hexon, penton and fiber proteins (Fields *et al.*, 1995 and White and Fenner, 1994).

There are approximately 50 serotypes of *adenoviridae* with the group C viruses (serotype 2 and 5) most extensively studied and developed for gene therapy applications. Initially, adenoviruses were evaluated as vectors for CFTR gene transfer to treat cystic fibrosis because of their natural tropism for pulmonary epithelial cells (Wilson, 1996). The advantages of using adenoviruses, as gene transfer vectors are that adenoviral vectors are highly efficient in transduction and can infect a broad range of differentiated non-dividing cells *in-vivo* (Curiel *et al.,* 1996). Moreover, adenoviral vectors can be grown to high titers (up to 10^{14} infectious particles per ml versus 10^{6} for retroviruses) of the purified recombinant virus, making for easy large-scale production (Russell and Peng, 2003).

The first-generation recombinant adenovirus is deleted in the early E1 regulatory region, rendering the virus unable to replicate. In addition, the E3 region is usually also deleted, to obtain room for the transgene. Thus, viral E1A proteins are provided *in trans* by a packaging cell line, usually Human Embryonic Kidney (HEK) 293 cells (Amalfitano and Parks, 2002).

A disadvantage of adenoviral vector-mediated gene transfer is that the transgene does not integrate into host genome but remains as an episome in the nucleus. As such, expression

is transient, lasting only days or weeks. However, the biggest disadvantage of adenoviral vectors, although somewhat remedied in subsequent generations, is their immunogenicity. The resultant toxicity associated with adenoviral vectors involves both the innate and adaptive immune response (Liu and Muruve, 2003). It has been observed that adenoviral gene transfer elicits a cytotoxic T cell response that destroys the transfected cells and induces humoral immunity due to elevated IgG and IgA antibodies (Van Ginkel *et al.,* 1995). These antiviral antibodies may neutralize the vector when administered a second time, and this may preclude repetitive dosage. In human trials, an acute local and systemic inflammatory response has been observed in vascular endothelium and lung tissue after adenoviral administration (Channon & George, 1997). To overcome these inflammatory and toxic problems, second- and third-generation adenoviruses have been created. In these vectors, more viral proteins encoding the sequence's E4 region (and possibly E2) are deleted to reduce the expression of viral proteins. On the other hand, this yields lower titers of the vector and makes it more vulnerable to contamination (Channon & George, 1997).

Human first-generation adenoviruses have been most widely used as *in-vivo* gene transfer vectors (Wilson 1996). They have been used for *in-vivo* human gene transfer into nasal and pulmonary cells (CFTR cDNA), vascular endothelial cells, kidney (Heikkilä *et al.,* 1996, Sukhatme *et al.,* 1997), heart, liver (Jaffe *et al.,* 1992), lung (Engelhardt *et al.,* 1993), central nervous system (Chen *et al.,* 1994, Simons *et al.,* 1999), renal cells (Chang *et al.,* 1995), muscle and hematopoietic cells and cancer cells (Cusack *et al.,* 1996).

Merrick *et al.,* (1996) compared the infectivity of a replication-defective type 5 recombinant adenovirus in various vascular endothelial cells in *in-vitro* culture, organ culture and *in-vivo*. They observed that, in culture, infectivity was good in both porcine and human vascular endothelial cells (up to 90% of the cells were infected at an adenovirus concentration of $1x10^{10}$ pfu/mL), but the *in situ* gene delivery into uninjured vascular

endothelium was markedly poorer, suggesting that some mechanisms other than vectors or target cells underlie the poorer *in-vivo* transduction efficiency. In a trial of gene transfer into malignant glioma cells via a catheter inserted into the tumor, Puumalainen *et al.,* (1998) compared the gene transfer efficiencies of retro- and adenoviruses. They found adenoviruses to be more efficient vectors for gene transfer into glioma cells than retroviruses, with transfection efficiencies of <0.01–11% and <0.01–4%, respectively (Puumalainen *et al.,* 1998).

Adeno-Associated Virus

Adeno-associated virus (AAV) is a small, non-enveloped, icosahedral, single-stranded DNA virus that can infect a wide range of tissues, including both dividing and non-dividing cells. Classified in the *Parvoviridae* family, AAV uses the heparan sulfate proteoglycan (HSPG) as the primary attachment receptor, with fibroblast growth factor receptor 1 (FGFR1) and integrin as co-receptors during viral attachment and endocytosis (Fields *et al.,* 1995 and White and Fenner, 1994). The normal lifecycle involves the infection and integration of viral genome into the host, where it stays latent. Unlike retroviral vectors, integration of the AAV genome is site-specific, to a locus AAVS1 on the q13.3-qter of human chromosome 19. Upon super-infection with other viruses such as adenovirus or herpes virus, AAV becomes active and replication occurs, facilitated by helper viral proteins (Lai *et al.,* 2002).

The AAV genome exists as a single-stranded DNA molecule of 4680 nucleotides with invert terminal repeats (ITRs) of 145 nucleotides flanking both ends. The ITRs are the only *cis*-acting elements required for replication, packaging and integration of AAV, provided that non-structural proteins *Rep* and capsid proteins *Cap* are provided *in trans* (Lai

et al., 2002). This allows for the replacement of the entire viral genome between the ITRs, pushing the packaging capacity to 4.9kb (Dong *et al.*, 1996). Since the default life cycle for AAVs is lysogenic, the production of rAAV vectors is technically more complicated than adenoviral vectors. First, co-transfection of two plasmids (rAAV transgene plasmid and AAV helper plasmid containing both *Rep* and *Cap* genes) results in the generation of lysogenic AAV. Upon transfection of a third plasmid encoding adenoviral helper proteins (such as E2A, E4orf6 and VA RNA transcription unit), generation of fully infectious rAAV vectors takes place. Purification by cesium chloride density centrifugation or heparin affinity chromatography enhances the viral titer up to 10^{14} particles per mL (Lai *et al.,* 2002).

The advantages of AAVs are that they are relatively non-toxic and non-immunogenic. The persistence of rAAV *in-vivo* can also be attributed by the lack of cellular immune responses elicited by this vector, which is devoid of all viral genes leaving the transgene product and the virus capsid as the only two sources of antigen (Lai *et al.,* 2002). This is particularly exemplified by the fact that the delivery of rAAV into a range of tissues has been followed by a general absence of inflammation (Conrad *et al.*, 1996, Xiao *et al.*, 1996, Dudus *et al.*, 1999). However, a humoral response to the AAV virion capsid generates neutralizing antibodies, which has been shown to prevent or greatly reduce the success of vector re-administration (Xiao *et al.*, 1999, Chirmule *et al.*, 2000.). Another advantage of using rAAV vectors is that transgene expression obtained is usually much longer than that of adenoviruses, due to the site-specific integration of transgene into the host genome. Murphy *et al.,* (1997) obtained 6-month normalization of hyperglycemia, insulin resistance and correction of the serum leptin level by recombinant AAV-leptin cDNA gene transfer in transgenic obese, type II-diabetic mice. Successful *in-vivo* reporter gene transfer into brain cells has been attained using AAV vectors, and transgene expression control was possible by simultaneous doxycycline administration (Haberman *et al.,* 1999). In addition, there are

reports of long-term expression following the delivery of AAV vectors into muscle (Fisher *et al.,* 1997), heart, brain (Haberman *et al.,* 1998), liver (Koeberl *et al.,* 1997) and lung (Flotte *et al.,* 1993).

Despite the long-term transgene expression and low immunogenicity, the main disadvantage of rAAV vectors are their small sizes (<5kb), which severely limits their application for larger gene products, such as the CFTR gene. Furthermore, the complicated process of generating rAAV vector makes large-scale production difficult and cost-prohibitive.

Herpes Simplex Virus

Relative to the previous examples of vectors, Herpes Simplex virus (HSV) is much larger in size. The herpes virus virion comprises four concentric layers: an inner *core*, surrounded by an icosahedral *capsid*, then an amorphous *tegument*, and finally an *envelope*. Within the *Herpesviridae* genus, the linear, double-stranded DNA genome can range from 125-229 kpb (White and Fenner, 1994). The virus is transmitted via direct contact with mucosal membranes. Subsequent to initial infection, the virus continually infiltrates and progresses to the nervous system. Herpes simplex virus has both lytic and latent phases of infection. During the lytic phase, herpes simplex virus infection is cytopathic, killing the cells it infects. During the latent phase, however, the virus can by maintained asymptomatically for a significant period of time (Fields *et al.*, 1995 and White and Fenner, 1994). Current development of herpes simplex as a gene therapy vector involves removing the replicative and cytotoxic genes. All genes essential for maintaining the virus in culture can be engineered into a packaging cell line called the amplicon system (Thomas *et al.,* 2003).

There are several advantages to developing HSV as a gene therapy vector. Its large genome can carry multiple, diverse therapeutic genes and can express them for an extended period of time. The virus can infect a wide variety of tissues including muscle, tumor, liver, pancreas, nerve, and lung cells. It has been used for gene transfer into neurons, brain tumors (Kennedy, 1997), various tumor cells and B cells (Levatte *et al.,* 1998). Carew *et al.,* (1998) reported effective IL-2 and lacZ reporter gene transfer by HSV amplicon vectors in murine squamous cell carcinomas after intra-arterial vector delivery. Nevertheless, the system's complexity mandates much more study of its biology before it can be contemplated as a viable vector in clinical studies.

Other Viral Vectors

Cytomegalovirus, baculovirus and poxvirus have also been investigated as gene transfer vectors. Vaccinia virus, a double-stranded DNA poxvirus, has been used for gene transfer into the lungs (Hogan *et al.,* 1998) and urinary bladder. Vaccinia virus recombinants were employed in intravesical instillation for gene transfer into normal bladder urothelium and transitional cell carcinoma cells *in-vivo* (Lee *et al.,* 1994). The authors suggested that this method could be used to introduce genes of immunogenic antigens and cytokines to elicit a host immunological response against superficial bladder cancer. A new lentiviral vector derived from equine infectious anemia virus (EIAV) has also been reported (Olsen, 1998). As these vectors are still in their infancy in terms of development, further characterization of their virology is needed to asses their suitability as gene therapy vectors. Furthermore, these new vectors must demonstrate clear advantages over existing viral vectors, especially in immunogenicity, length of transgene expression, potential for insertional mutagenesis and ease of vector generation.

Non-Viral Vectors

Eukaryotic cells can, under suitable conditions, take up exogenous DNA and relay it, at least in part, to the nucleus. This process, however, is usually insufficient for gene therapy, and a wide variety of gene-transfer-facilitating methods other than immunogenic viral vectors have therefore been developed. These methods involve: 1) liposomes, 2) calcium phosphate, 3) microinjection, 4) electroporation, 5) polycations, and 6) particle bombardment (Unger, 1997). The synthetic methods designed for gene transfer are extremely varied. To put it simply, these vectors generally use natural mechanisms of mammalian cells for the uptake and intracellular transport of macromolecules. Molecular aggregates are generally formed with plasmid DNA, which subsequently bind to cell surfaces and trigger endocytosis of the vector for the transgene to be transported into the cell nucleus. The major advantage of these methods is that they are relatively non-immunogenic, except for the possible immune response which the transgene itself may elicit in the recipient (Schagen *et al.*, 2004).

Non-viral vectors make use of physical methods of gene transfer. These methods can largely avoid the problems of immunogenicity and inflammation that plague viral vectors, but suffer from poor and transient expression of the therapeutic gene. It is also more difficult to target expression to specific cells and tissues. As a possible solution, the application of non-viral vectors for use in *ex-vivo* gene delivery is a promising approach, as evidenced by the recent success expressing recombinant Factor VIII for the treatment of hemophilia in mice (Lin *et al,* 2002).

Naked DNA

Gene expression has been demonstrated using naked synthetic or plasmid DNA after injection of the DNA into cardiac muscle, parenchymal, thymus and skin cells (Budker *et al.*, 1996b). The most effective delivery route for naked DNA, however, is injection into skeletal muscle. Multiple injections of naked DNA can improve therapeutic gene expression, but since integration occurs rarely with plasmid DNA, long-term expression of the transgene rarely occurs (Hengge *et al.*, 2001). Naked DNA has been employed as a gene therapy for both preventative and therapeutic purposes in terms of vaccination, termed "DNA vaccines". Instead of site injection of protein antigens, DNA is injected intramuscularly, where the expression of the transgene coding for the antigen effectively elicits the requisite immune response (Wahren, 1996). The main advantages of naked DNA are unlimited therapeutic gene length and ability to inject directly into a patient. However, aside from intramuscular injection, the use of naked DNA is extremely inefficient, and thus, its scope is limited to a narrow range of applications. Furthermore, there is no established mechanism of naked DNA transfer into the nucleus, nor is it known why injection into striated primate muscle is more successful than other sites of delivery. Nevertheless, intramuscular DNA vaccines are currently being investigated to prevent cancer and infectious diseases (Gene Therapy Clinical Trials Worldwide Trial IDs: EG-001 for Hepatocellular Carcinomas, US-254 for Metastatic Melanoma, US-392 for Non-Hodgkin's B-Cell Lymphoma, US-555 for Hormone-Refractory Prostate Cancer, US-573 for Melanoma).

Calcium Phosphate Precipitation

Calcium phosphate precipitation has been used as a method of transfection of plasmid DNA into cultured cells, such as hepatocyte cell lines, since the 1970s. But in *in-vivo*

applications, the efficiency has not been very high, with transfection frequencies being less than 1% (Chang & Wu 1994). This method was primarily reported in the first gene transfer trial by Graham and Van Der Eb (1973). Calcium phosphate forms insoluble precipitates with plasmid DNA and aggregates on the surface of the target cell, resulting in their endocytosis. The insoluble precipitates demonstrated certain limited resistance to cytosolic nucleases, which can present a major barrier for non-viral gene transfer. Unlike naked DNA, calcium phosphate DNA co-precipitates gain access to the nucleus via endosomal-lysosomal pathways. Entry into the nucleus has been hypothesized to occur after the dissolution of the nuclear membrane during mitosis, but conflicting data have also reported the irrelevance of mitosis to transfection success (Dean, 1997). Due to the limited transfection efficiency via this method, most *in-vivo* gene transfer investigations have abandoned the calcium phosphate method in favor of other non-viral means. Recently, however, it has been used together with the adenoviral vector to enhance transfection efficiency in the airway epithelial cells (Lee *et al.*, 1999 and Walters and Welsh, 1999).

Lipoplexes

Alternatively, DNA can be attached to positively charged lipids to accommodate gene transfer into the patient's cells. Liposomes are fatty acid vesicles that have the ability to encapsulate DNA. Cationic lipids are positively charged fatty acids that interact with negatively charged phosphate backbone DNA, forming a stable complex. The term, lipoplex, has gained popularity to describe liposome-DNA complexes and has been used to encompass all variants of liposome-based transfection techniques. The first commercially available cationic lipid was used as an *in-vitro* transfection tool (Felgner *et al.*, 1987), but all three types of lipids (pH sensitive, anionic and cationic liposomes) are also capable of *in-vivo* gene

delivery. Cationic liposomes complexed with plasmid DNA have transfected cells when injected intravenously, subcutaneously, and by aerosol delivery. Although all three types of liposomes have been described in successful gene transfer, pH-sensitive and anionic complexes are less efficient than their cationic counterparts (Zhdanov *et al.*, 2002). Currently, the primary liposome-based transfection methods are based on either monocationic or polycationic lipids.

The entry of the synthetic cationic lipid bilayers complexed to the negatively charged plasmid to be transferred into the target cell occurs by a cell-membrane fusion event or endocytosis (Felgner *et al.,* 1987). Positively charged liposome DNA complexes are capable of significant changes when injected into the blood stream. Liposomes can protect the therapeutic DNA from degradation in the blood stream. Expressions of hormones, cytokines, or coagulation factors have also been successful with intravenous delivery of liposome DNA complexes.

Plasmid-liposome complexes have many advantages as gene transfer vectors. They are relatively non-immunogenic, which makes them suitable for safe repeatable dosing. They also possess a low risk of integration into patient DNA, and successful transient expression of plasmid DNA vectors of virtually unlimited size has been demonstrated. Moreover, liposomes are inexpensive to manufacture in large quantities, which makes them ideal as a broad-based vector for gene based therapeutics. Lipoplex mediated gene transfer has been successfully used for gene transfer into the pancreas (Schmid *et al.,* 1998), lung (Alton *et al.,* 1993, Wheeler *et al.* 1996), arterial (Laitinen *et al.,* 1997) and liver cells (Baru *et al.,* 1995). Depending on the application, the immune response induced by foreign DNA can have a therapeutic action aside from any immune response to the protein produced from the inserted gene (Freeman *et al.*, 1996).

The disadvantages of employing lipid-based gene transfer vectors for *in-vivo* applications can include toxicity for certain lipids and the serum instability of cationic lipoplexes. However, their main disadvantage remains to be their relatively low transfection efficiency compared to viral vectors. Currently, their transfection efficiency has been improved by simultaneous delivery of agents that prevent DNA degradation within endosomes (Budker *et al.,* 1996a). Also, the liposome-mediated gene transfer efficiency has been improved by complexing viral particles with liposomes, such as the Sendai (HVJ) virus (Tomita *et al.,* 1992, Yonemitsu *et al.,* 1997). Using this method, Tomita *et al.,* (1992) reported renal gene transfer into 15% of glomerular cells after intra-arterial infusion of vectors four days after treatment. By conjugating liposomes with antibodies or ligands, better targeted lipofection can also be achieved.

Polyplexes

One of the earliest agents used for non-viral gene transfer, DEAE-dextran (Vaheri and Pagano, 1965) is a polycationic polymer-based DNA complexing agent. Structurally, positively charged DEAE-dextran binds to the negatively charged phosphate backbone of the DNA, causing aggregation. Polyplexes, once endocytosed, are purportedly released from endosomal-lysosomal degradation via the "proton-sponge mechanism" into the cytosol. Due to cytotoxicity and low transfection efficiency initially associated with DEAE-dextran, DNA-polyplexes did not become widely used until the introduction of another polycationic polymer - polyethyleneimine (PEI). Currently, the term "polyplex" has evolved to encompass a variety of polycationic species, including numerous naturally occurring proteins such as histones (Balicki *et al.*, 2000) and cationized human serum albumin (Fischer, 2001), as well as aminopolysaccharides such as chitosan (Leong *et al.,* 1998 and Borchard, 2001).

Polyplexes also include a variety of synthetic peptides such as poly-L-lysine, poly-L-ornithine (Pouton *et al.*, 1998) and poly (4-hydroxy-L-proline ester) (Lin *et al.*, 1999), as well as polyamines such PEI, polypropyleneimine and polyamidoamine dendrimers (Qin *et al.*, 1998). Finally, both linear and dendritic poly (β-aminoesters) have also been employed for DNA-polyplex gene transfer. Recently, PEI has been used for gene transfer into lung cells (Ferrari *et al.,* 1997, Goula *et al.,* 1998).

The main advantage of using polyplexes as gene delivery vectors is the structural flexibility and ease of modification. A variety of receptor targeting ligands can be chemically attached to a current polyplex to enhance specificity. Moreover, various structural/activity relationships are being studied to yield the modifications needed for the development of the next generation of polyplexes to enhance transfection efficiency while reducing toxicity (Wagner, 2004).

Electroporation

When cells are incubated in a DNA solution, the application of a pulse of electrical current can potentially create transient holes in the cell membrane, through which DNA can be transferred (Weaver, 1993). The technique was adapted from *in-vitro* approaches designed to transfect cells with DNA plasmids, and been extensively exploited to transfer genes directly to muscle to deliver therapeutic genes and as a strategy for genetic immunization. The use of electroporation has demonstrated success in mammary tumor cells (Wells *et al.*, 2000), lung cells (Dean *et al.*, 2003) and *in-vivo* melanoma (Kishida *et al.*, 2001) gene transfer. The advantage of electroporation is its relative transfection efficiency over other non-viral means. However, the use of electroporation for *in-vivo* treatment is

extremely limited due to equipment constraints, coupled with the massive cell death that occurs post-treatment.

Ballistic Methods

"Particle Bombardment" was first developed to transfer genes into plants cells, but has been adapted for use in mammalian cells and living tissues. The plasmid DNA is first coated with a metal such as tungsten or gold (Biewenga *et al.,* 1997). These particles are then accelerated by a particular driving force, such as a high-voltage discharge between two electrodes or helium discharge, which propel the coated DNA particles into the cell. DNA particles are literally driven across the plasma membrane, which is why this technique is often referred to as the "Gene Gun".

Ultimately, transfection efficiency depends on the combination of the ballistic parameters and the characteristics of the target tissue. This technique has been successful in delivering genes to brain tissue (Jiao *et al.,* 1993), skin, muscle (Zelenin *et al.,* 1997), liver (Yang *et al.*, 1990) and numerous other organs. Moreover, it has also been shown to be an efficient means of gene transfer into cancer cells (Zhang *et al.*, 2002).

The main advantage for employing this technique is that it is a mechanical way to transfer a gene across the plasma membrane, which presumably makes transfection efficiency less dependent on the characteristics of the target cell. Particle acceleration-mediated transfection is generally less effective than viral methods, but more effective than lipofection or calcium phosphate precipitation. However, when Guo *et al.,* (1996) compared particle bombardment, lipofection, calcium phosphate precipitation and retroviral gene transfer *in-vitro* for the reporters *lacZ* and luciferase into rat oligodendrocytes, they found

that the most effective of these four methods was particle bombardment-mediated transfection with gene gun accelerated DNA, coated in 0.95 μm gold particles.

The main disadvantage of particle bombardment is the harsh nature of the process. Its initial applications in plants were particularly suitable because it overcame a major structural barrier – the plant cell wall. However, mammalian cells lack this structural feature that, in certain ways, protects the plant cell from extensive damage resulting from the bombarding particles. Thus, due to the nature of the delivery, a considerable amount of cellular damage is inflicted upon the transfected mammalian tissue(s). Various cellular components, including cellular DNA, are damaged mechanically when accelerated particles are showered indiscriminately onto the cells. Not all cells can withstand this type of gene transfer; thus, it is mainly used for genetic immunization into skeletal muscles.

Mammalian Transfection thru Non-Viral Means

Factors Affecting Transfection Efficiency

Almost as far back as the classic experiments performed by Cohen *et al.,* in the 1970's, there have been efforts to enhance the uptake of exogenous DNA. Transfection efficiency, as observed by numerous researchers, was largely dependent on various general, cellular (Reston *et al.*, 1995) and mechanistic factors (Kamiya *et al.,* 2001). In the hopes of developing a highly efficient, non-immunogenic and universal vector for DNA delivery, researchers are investigating the cellular and mechanistic factors affecting transfection efficiency. However, results have been far from being straightforward. As the numerous optimization variables are investigated, more factors revealed themselves to be critical for the success of each transfection experiment. Worse yet, optimization protocols and transfection

enhancing agents are largely cell-type or transfection-method dependent, making the process much more complicated than was once envisioned. Through the years, these investigations, while failing to produce a truly universal vector, have contributed to the fundamental understanding of the transfection mechanism.

Factors affecting transfection can be segregated into three groups: general, cellular and mechanistic factors. General factors include various guidelines that are common to all transfection protocols, such as the quality of transfected DNA. Cellular factors constitute the physiological variables that affect transfection efficiency. These factors can be further sub-categorized into physiological barriers (such plasma membrane, endosomal-lysosomal compartments and nuclear membrane) and cellular conditions (such as rate of cell proliferation, cell type dependence and apoptotic propensity). Mechanistic factors, on the other hand, are transfection method-dependent factors, often directed at enhancing a certain choke point in the transfection process. These are further segregated into optimization conditions (such as size of DNA-polycationic aggregates, zeta potential and serum stability) and transfection enhancing agents (such as glycerol, cortisol and sodium glycolate). These factors are extensively reviewed below.

General Factors Affecting Transfection Efficiency

Quality of DNA

As is generally recommended by a variety of protocols, the quality of DNA has been deemed to be one of the most important factors determining transfection efficiency (Promega transfection guide and Schenborn and Goiffon, 2000). Aside from the usual ethanol precipitation, DNA is recommended to be purified by the cesium chloride equilibrium centrifugation method, with the final A_{260}:A_{280} ratio of 1.8 or greater being acceptable for

transfection. Aside from being able to accurately measure DNA concentration and subsequently optimize transfection efficiency based on an accurate DNA concentration, the purity of DNA is deemed important because any bacterial contaminants are considered to inhibit transfection efficiency (Weber *et al.*, 1995). However, a recent report by Wright *et al.,* (2003) demonstrated the use of partially purified DNA in transfecting HEK293-EBNA cells, with transfection efficiency as judged by levels of recombinant protein expression to be equivalent to that of pure DNA. In other reports, partially purified DNA actually resulted in an enhancement of transfection efficiency via electroporation in BALB/c 3T3 cells (Tatsuka *et al.,* 1995). On the other hand, based on observations in our own laboratory, transfection of impure DNA resulted in high levels of toxicity to HeLa and MDBK cells.

Cellular Factors Affecting Transfection Efficiency

Physiological Barriers

There are numerous intra- and extracellular barriers to the introduction of therapeutic genes via non-viral means. *In-vivo* transfection must be able to survive systemic circulation to reach its intended target. Numerous cells of the immune system have the ability to sequester the transfection agent, elicit an immune response to the transgene product and destroy transformed cells. Assuming the DNA reaches its intended target, there are more hurdles to cross. To simplify our discussion, *in-vitro* transfection also encounters some of these hurdles. For successful transfection to occur, the transfer of DNA into a cell requires for it to pass through various cellular compartments and enter the nucleus for transcription. Through the numerous intermediary stages, a portion of the transfected DNA is eliminated. By the time the DNA traverses all of the cellular compartments, including the plasma membrane, endosomes, lysosomes, the cytosol and the nuclear membrane, only a small

amount of the initially transfected DNA survives to make its way into the nucleus. It has been estimated that up to 100,000 copies of plasmid can be taken up per cell (Batard *et al.*, 2001). However, 3000 plasmids must survive in the cytosol for transgene expression to occur (Batard *et al.*, 2001), which explains why transfection suffers from such low efficiency.

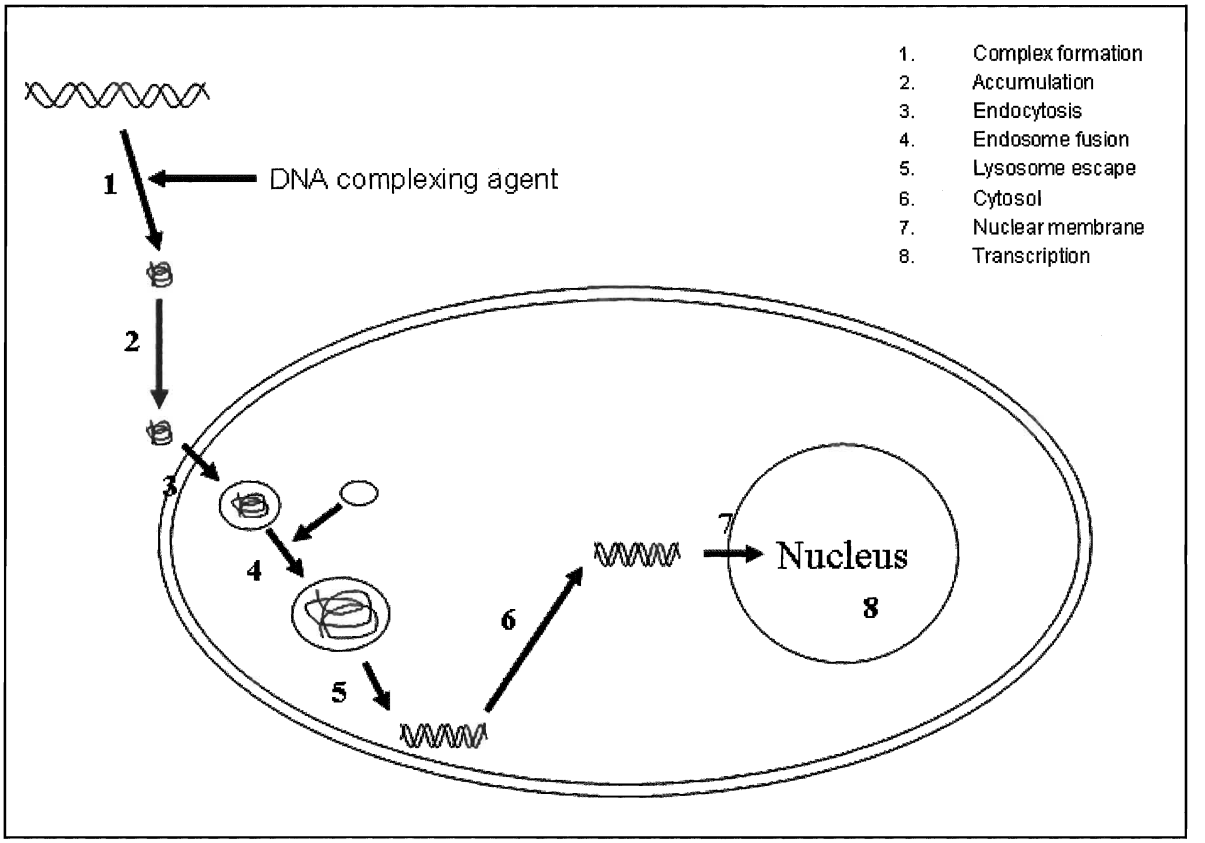


Figure 1: The path of exogenous DNA to the nucleus and the various chokepoints during transfection. 1. Complex formation requires the exogenous DNA to be compacted with the DNA complex formation. 2. Accumulation of DNA must occur onto the surface of the cell. 3. DNA-complex must be efficiently endocytosed. 4. Endo-lysosomal compartments contain various DNases that degrade exogenous DNA. 5. Release of the DNA from the complexation agent as well as escape from lysosomal compartments must occur efficiently. 6. A host of DNases in the cytosol can degrade exogenous DNA effectively. 7. Nuclear membrane presents the last major barrier to transgene transcription in the nucleus 8.

Internalization of Exogenous DNA

Before DNA is internalized, whether it is coupled to a viral or non-viral carrier, it must accumulate near the surface of the plasma membrane. Since DNA is a polyanion, naked DNA does not readily bind to the cellular membrane, whose net charge is also negative. Thus, the pre-endocytic accumulation of DNA represents a significant barrier for naked DNA transfection. This notion is supported by the observation that naked DNA transfection is an extremely inefficient process without the help of vectors.

Internalization of plasmid DNA can be achieved by either fluid phase-, adsorptive-, or receptor-mediated endocytosis (Wattiaux *et al.*, 2000). It is thought that transfected DNA enters the cell through an adsorptive endocytic process. Although a large amount of DNA is generally transfected, only 5% of the DNA is ever endocytosed (Batard *et al.*, 2001). While there are attempts to enhance other means of endocytosis, such as including transferrin with lipofectin during transfection to enhance receptor-mediated endocytosis, evidence suggests that endocytosis is most likely not the rate-limiting factor in determining the overall outcome in transfection (Tseng *et al.*, 1997). Furthermore, Coonrod *et al.* (1997) concluded that endocytosis could not account for the two magnitudes of difference in transfection efficiency between the human immortalized cell line, HeLa, and fibroblasts after observing nearly similar rates of endocytosis between the two cell lines. However, there are other studies correlating endocytic rate and transfection efficiency in Chinese hamster ovary (CHO) cells (Batard *et al.*, 2001). Moreover, transfection efficiency was enhanced in CHO cells when DNA silica nanoparticles were physically concentrated by centrifugation, suggesting the importance of the association of DNA with the plasma membrane during transfection (Luo and Saltzman, 2000a). Therefore, it seems that internalization can potentially represent a

significant barrier to transfection in a cell-type specific manner and may also vary according to delivery methods.

Endosomal and Lysosomal Compartments

Regardless of the route of entry across the plasma membrane (endocytosis, lipid fusion or electroporation), transfected DNA follows a common route to the nucleus through vesicular trafficking in the endosome/lysosome system (Coonrod *et al.*, 1997). For successful transfection to occur, a prerequisite is that a viable proportion of intact DNA molecules should escape endosomal or lysosomal destruction en route to the cytosol and eventually the nucleus. Therefore, factors and/or agents affecting the endosomal and lysosomal compartments could potentially either facilitate the release of plasma DNA into the cytosol, or protect these molecules from degradation by lysosomal nucleases (Neves *et al.*, 1999), thereby influence transfection efficiency.

At 30 minutes after DNA is transfected, plasmid DNA is internalized and finds its way into endosomal compartments. Vesicle transport is evident by the visualization of a faint granular centripetal cytoplasmic pattern that is largely distinct from the lysosome and nucleus (Coonrod *et al.*, 1997). During endosomal transport, DNA remains relatively unscathed, as little nuclease activity has been found in the endosomal compartments. Peptidases, on the other hand, are found in abundance. While the roles of the peptidases are unknown, they have been postulated to play an essential role in the activation of lysosomal nucleases after the fusion of endosomes with lysosomes (Wattiaux *et al.*, 2000). During the fusion and endosome processing events, endo-lysosomal compartments undergo internal acidification, during which pH-sensitive nucleases are activated to destroy the transfected DNA. What adds to confusion is that there is evidence suggesting that vector hydrolysis in the endosome is a prerequisite for the endocytosed DNA to quickly reach the lysosome

(Wattiaux *et al.*, 2000). Therefore, greater vector resistance to hydrolysis by peptidases such as cathepsin -B and -C may extend transfected DNA longevity by delaying endosome-lysosome fusion and facilitating the release of plasmid DNA from endosomal compartments into the cytosol.

Various non-viral vectors act to enhance transfection efficiency by mediating early escape from the endosomal compartments. A common practice during calcium phosphate co-precipitation transfection is to add certain reagents, such as glycerol or dimethylsulfoxide (DMSO), in a post-transfection "shock" treatment. These reagents are found to destabilize membrane integrity which may allow for plasmid DNA to escape lysosomal destruction (Frost and Williams, 1978). Other popular non-viral vectors, such as polyethyleneimine, can potentially cause the swelling and rupture of the organelles by sequestering surrounding protons and their counter-ions, creating an osmotic imbalance.

Another possible mechanism for enhancing plasmid DNA longevity in the endosomal/lysosomal compartments is to prevent the internal acidification and processing of the lysosomal nucleases. In fact, viral capsid proteins offer protection against nucleases by shielding the transgene from the endosomal/lysosomal environment. While non-viral vectors usually form extensive complexes with the DNA, offering some protection against nucleases by changing DNA conformation, the level of protection may not always be sufficient (Batard, 2001). Chloroquine is sometimes used as a means of enhancing transfection efficiency during calcium phosphate transfection. It has been shown to neutralize lysosomal pH, inhibit proteases and presumably aids in transfection by disrupting nuclease maturation. However, chloroquine does not always enhance transfection efficiency, and the effects seem to be cell-type specific (Coonrod *et al.*, 1997).

Yet another approach to enhance transfection is by inhibiting nuclease activity. DNase II is an endonuclease with an acidic pH optimum that was identified in the lysosomal

fraction of porcine spleen (Liao *et al.*, 1989). Since its discovery, DNase II was deemed to be of paramount importance in transfection. This was demonstrated by the 10-fold enhancement of reporter expression in H441 cells when transfection occurred in the presence of DMI-2, a fungal metabolite known to inhibit DNase II (Ross *et al.*, 1998). Moreover, peptidal inhibitor ID2, discovered based on library screening, significantly enhanced transfection efficiency by polycationic compounds DOTAP and PEI (Sperinde *et al.*, 2001). However, the story does not end there. As reported by Ross *et al.* (1998), the inclusion of DMI-2 during calcium phosphate transfection in the same cell line did not enhance transfection efficiency. These results seem to indicate that the extent of lysosomal nuclease to transfection efficiency may depend on delivery methods.

DNase II

DNase II, also known as acid DNase, derives its name from the acidic pH optimum for nuclease activity (pH 4.5-5.0). Its activity was found to have a ubiquitous tissue distribution (Cordonnier and Bernardi, 1968). Unlike other DNases, DNase II does not require the presence of divalent cations for nuclease activity to occur. Furthermore, certain divalent cations, such as Zn^{2+} and Cu^{2+} strongly inhibit DNase II activity (Bernardi, 1971; Hevelone and Hartman, 1988; Lyon and Aguilera, 1997). Due to the pH optimum of DNase II, it was first proposed and later confirmed to be most active in lysosomal compartments (Liao *et al.*, 1989). Presumably, the internal acidification that occurs during the fusion of the endosome with the lysosome activates the enzyme, which can present a potential barrier to transfection (Howell *et al.*, 2003).

While the physiological role of DNase II has not been fully elucidated, there is evidence that DNase II is associated with nuclear fragmentation during apoptosis. Recently, Krieser *et al.* (2002) has shown the importance of DNase II during the phagocytic phase of

apoptosis. Homozygous knockout mice resulted in perinatal lethality, presumably due to the improper accumulation of apoptotic nuclei during fetal development. In the same publication, the authors suggested that DNase II activity loss might contribute to the development of systemic lupus erythematosus.

Cytosolic Degradation

After endocytosis, it was generally assumed that the lysosomal compartment was the major site of plasmid degradation after the discovery of DNase II, and the release of plasmid DNA into the cytosol represented a "safe zone" before traversing to the nucleus, with little consideration for the rate of cytosolic transportation. However, evidence now suggests that plasmid DNA is susceptible to degradation by cytosolic nucleases (Lechardeur *et al.*, 1999). Furthermore, this nuclease is constitutively present in the cytosol, rather than by nuclease either liberated from organelles or activated upon the introduction of DNA. Further characterization indicated the pH optimum of these nucleases to be 7-8, which would group them into the DNase I family of neutral DNases. Members of DNase I family of DNases include DNase I, DNase X, DNase γ and DNase 1L2, of which DNase I and DNase γ are the most extensively characterized.

DNase I

DNase I is one of the most characterized mammalian endonucleases. It was originally identified as a pancreatic enzyme that catalyzed DNA hydrolysis in the presence of appropriate divalent cations (Lakowski, 1971). DNase I has been purified from various mammalian tissues and body fluids, and its characterization has revealed that DNase I is an endonuclease that exerts its full activity in the presence of both Ca^{2+} and Mg^{2+} under neutral conditions (Price, 1975 and Campbell and Jackson, 1980). Among other endonucleases

discovered with the similar primary structures, numerous members of endonucleases are now classed into the DNase I family of DNases. These members include DNase I, DNAS1L2, DNase γ/DNAS1L3/LS-DNase/DNase Y, and DNase X/Xib, with both DNase I and DNase γ being the most extensively characterized (Shiokawa *et al.*, 2000). Biochemically speaking, DNase I is active in the presence of a single bivalent cation, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+} or Co^{2+} (Liu *et al.*, 1999). Aurintricarboxylic acid (ATA) and monomeric actin (G-actin) are known inhibitors of DNase I (Lacks, 1981).

While much is known about the biochemical characteristics, the physiological function of DNase I has never been addressed fully. DNase I have been associated with a wide range of functions in recombination, maintenance of genetic stability and transformation (Baranovskii *et al.*, 2004). Recently, Napirei *et al.,* (2000) and Walport (2000) reported a putative scavenger role for DNase I. DNase I-deficient mice demonstrated classical symptoms of developing systemic lupus erythematosus such as the presence of anti-nuclear antibodies, the deposition of immune complexes in glomeruli and full-blown glomerulonephritis in a DNase I dose-dependent manner. The authors suggested that these symptoms are the result of inadequate elimination of DNA from circulating dead cells, resulting in the development of anti-nuclear antibodies directed against naked DNA and entire nucleosomes.

DNase γ

DNase γ was originally identified as an endonuclease present in the nuclei of rat thymocytes, and its physiological role has been suggested to be the catalysis of nucleosomal DNA fragmentation during apoptosis (Tanuma and Shiokawa, 1994 and Shiokawa *et al.*, 1994 and 2002). Recently, DNase γ has also been suggested to constitute a potential barrier

to liposomal transfection, stemming from the observation that non-viral vectors offer some protection against DNase I (Wilber *et al.*, 2002). DNase γ is a Ca^{2+} and Mg^{2+} dependent neutral endonuclease that exists specifically in lymphoid organs such as thymus, spleen, and lymph node. In terms of biochemical characteristics, DNase γ is strongly inhibited by Zn^{2+} (Shiokawa *et al.*, 1997) and ATA, while retaining activity in the presence of G-actin at 100μg/mL (Shiokawa *et al.*, 1997).

Nuclear Membrane and Nuclear Purging

Approximately one hour post-transfection, plasmid DNA accumulates in the perinuclear region (Coonrod *et al.*, 1997). Before gaining entry into the nucleus, the final physical barrier that must be traversed is the nuclear membrane. It has been postulated, based on observations that cell cycle synchronized cells often demonstrated enhanced transfection efficiency around the time of mitosis, that plasmid DNA can gain access to the nucleus during the dissolution of the nuclear membrane. More recently, however, it has been found that DNA enters the nucleus through specialized structures known as the nuclear pore complex (Dean, 1997). There are several factors affecting the rate of nuclear import of DNA. First, the size of the transfected DNA has a detrimental effect on the transport kinetics. DNA Fragments less than 1kb can enter the nucleus readily (Hagstrom *et al.*, 1997). However, for plasmids bigger than 1kb, active uptake of DNA through the nuclear pore is a slow and energy dependent process (Neves *et al.*, 1999). Secondly, the presence of nuclear localization signals (NLS) may facilitate nuclear import. NLS sequences bind to importin α in the cytoplasm. The resulting complex binds to the nuclear port and is translocated through the pore in a mechanism involving energy, the small GTPase Ran and other proteins (Neves *et al.*, 1999).

In nonviral gene transfer into non-dividing cells, the entry of plasmid DNA from the cytoplasm into the nucleus is a major limiting step (Wolff, 1997 and Escriou *et al.*, 1998). This was further supported by the fact that it takes approximately an additional hour before exogenous DNA enters the nucleus where it continues to accumulate before peaking at 8 hours post-transfection (Coonrod *et al.*, 1997).

Gaining entry into the nucleus, however, does not guarantee transgene expression. Non-integrated exogenous DNA is quickly purged in the nucleus. Coonrod *et al.* (1997) observed that while internalization kinetics and plasmid stability were comparable between HeLa cells and fibroblasts, the authors attributed the large difference in transfection efficiency to the active removal of DNA from the nucleus. They have, in turn, termed this process as "nuclear purging". Furthermore, without a mammalian origin of replication, plasmid DNA is slowly diluted out with each cell division. Moreover, integration of exogenous DNA into a transcriptionally inactive region by histone modification, ubiquitination, acetylation, methylation and repeat-induced gene silencing mechanisms can result in lack of observable long-term expression.

The grim truth is that only a fraction of transfected DNA ever makes its way to the nucleus. It has been suggested that only 5% of transfected plasmid is ever internalized (Batard *et al.*, 2001). Of that, a large fraction is degraded in the endosomal/lysosomal compartments. Moreover, only 0.1% of the cytosolic plasmid makes its way into the nucleus. On top of these problems, nuclear purging of exogenous DNA seems to be an effective mechanism, at least for primary cultures of fibroblasts, in eliminating transfected plasmids (Coonrod *et al.*, 1997). Using intracellular injection of DNA, as high as $1*10^6$ copies are required to reach the cytoplasm for transgene expression to eventually occur makes it obvious why non-viral methods suffer from such low efficiency (Utvik *et al.*, 1999).

Cellular Conditions

As observed in numerous laboratories, including our own, transfection efficiency often depends on the cellular conditions of which the cell cultures are kept. The degree of transfection variability can simply be affected by trivial matter such as the rate at which cells are sub-cultured and the amount of serum present in the culture media. Although these variables are generally determined empirically, the fundamental reasons for doing so remain far from being fully understood. Generally, cellular conditions can have large effects on the outcome of transfection experiments. These include: cell type dependence (Lin *et al.*, 1994), cell proliferation (Fasbender *et al.*, 1997), and apoptotic propensity (Kim *et al.*, 2002).

Cell Type Dependence

Numerous reports have documented the cell-type dependence of transfection efficiency (Lin *et al.*, 1994). Regardless of transfection methods, certain cell lines refuse to be transfected. Generally, primary cultures, such as fibroblasts and HUVEC cells, are notoriously difficult to transfect, whereas immortalized cell lines, such as HEK 293 and HeLa are more prone to taking up and expressing transgenic DNA. Whether the transformation process affects the cell line's propensity to be transfected is a matter of contention. Pampinella *et al.* (2002) reported that primary myoblasts and established myoblasts exhibit a difference in liposome-mediated transfection not by differential uptake of lipopolyplexes, nor by differential stability of the plasmid DNA in the endo-lysosomal or cytoplasmic compartments. Instead, the slower delivery of lipopolyplexes to the lysosomal compartment is one of the factors responsible for the pronounced transfectability of established myoblasts (Pampinella *et al.*, 2002).

Cell Proliferation/Cell Density

As a general guideline, cultured mammalian cells are usually plated at a low density the day prior to transfection by non-viral means. Establishing a population of rapidly dividing cells was deemed important, as it was a widely held belief that transfection efficiency correlated to the number of cells actively undergoing mitotic cell division. This was particularly demonstrated for retroviral gene transfer (Springett *et al.*, 1989), liposome-based techniques (Mortimer *et al.*, 1999) and peptide-based gene delivery systems (Wilke *et al.*, 1996). The argument was that once successful internalization occurs, nuclear entry of exogenous DNA is facilitated by the dissolution of the nuclear membrane during mitosis, allowing for the observed increase in transgene expression of rapidly proliferating cells (Fasbender *et al.*, 1997). Numerous attempts at verifying this conclusion by using cell-cycle synchronizing agents, such as hydroxyurea and thymidine, have generated dubious results (personal observation), and the approach of using these agents to associate cell cycle effects and transfection has come under increasing criticism due to the pleotrophic effects of these agents (Rodriguez and Flemington, 1999). However, other attempts to enhance transfection by stimulating cell proliferation, such as the use of mitogens, have also generated varying levels of success in enhancing transfection efficiency (Somasundaram *et al.,* 1992 and Ohmiya *et al.*, 2002). Recently, however, the correlation between cell cycle effects and transfection efficiency, especially the condition that mitosis and dissolution of nuclear membrane is a prerequisite for successful transfection, has been steadfastly challenged based on observations that adenoviral or lentiviral gene-transfers do not require cell division for efficient gene transfer (Greber *et al.*, 1993 and Bukrinsky *et al.*, 1993). The increasing consensus is that exogenous DNA has the potential to gain access to the nucleus by active transport processes through the nuclear pore complex, provided that the delivery method takes advantage of the nuclear localizing signals.

Presence of Serum

One of the problematic areas, especially when lipoplexes are employed during transfection, is that the presence of serum proteins significantly reduces transgene expression (Felgner *et al.*, 1987). It is thought that serum proteins interact with the membrane bilayers of liposomes, destabilizing them (Bonte and Juliano, 1986 and Li *et al.*, 1999). Furthermore, serum proteins can potentially block lipoplex association with cell membranes (Yang and Huang, 1997), reducing their ability to aggregate at the membrane (Hui *et al.*, 1996) and may also lessen uptake into endosomal vesicles (Lewis *et al.*, 1996). As was demonstrated by Felgner *et al.,* (1987), transfection of mouse L cells with DOTMA is inhibited by serum-containing growth medium, reducing the CAT activity to 5% of control. However, this serum-inhibition seemed to be cell-type dependent as well, as Brunette *et al.,* (1992) reported no inhibition in transfection efficiency in the presence of serum for CV-1 and murine erythroleukemia (MEL) cells. As a precautionary step, however, most commercially available protocols suggest the use of serum-free media to achieve maximal transfection efficiency.

The use of serum-free media during transfection, as a solution to serum-mediated inhibition, bears two notable difficulties. First, in the pursuit of developing an *in-vivo* gene delivery tool, serum inhibition presents a significant hurdle towards achieving that goal. Secondly, certain cell lines, such as C2C12 cells, respond adversely to the lack of serum exposure to prolong periods of incubation in serum-free media often demonstrated notable levels of toxicity (Dodds *et al.,* 1998).

To remedy the problem, ever-newer formulations of lipoplexes have been designed to minimize the inhibitory effects of serum. By comparison, commercially available cationic lipids DOSPER retained the ability to transfect C2C12 cells in the presence of serum over

Lipofectamine (Dodds *et al.,* 1998). Yang and Huang (1997) have reported that increasing the charge ratio of cationic liposome-DNA complexes can alleviate serum inhibition of lipoplex transfection efficiency. Additions of other additives to enhance serum stability of lipoplexes have also shown to enhance transfection efficiency. The use of polycation polybrene (Abe *et al.,* 1998), vesicular stomatitis virus G glycoprotein (VSV-G) (Abe *et al.,* 1998b), cholesterol (Crook *et al.*, 1998) and transferrin (Tros de Ilarduya and Düzgüneş, 2000) as additives during lipoplex-mediated transfection was observed to abrogate serum-mediated inhibition.

Apoptotic Propensity

Another possible scenario resulting in poor transfection efficiency is not the sole result of inefficient gene delivery. Rather, successful gene delivery occurs but most transfected cells subsequently die as a result of the toxicity of the transfection procedure. Thus, only the cells that survive are tabulated as transfection positive. While certain cell lines, such as HEK-293, are less prone to the toxicity involved during transfection, numerous reports have noted cell death as one of the main choke points in achieving efficient transfection (Kim *et al.*, 2002 and Rodriguez and Flemington, 1999). The toxicity can stem from a variety of sources, including exogenous DNA, transfection method and bacterial contaminants during plasmid DNA preparation. The resulting toxicity can trigger apoptosis, resulting in the death of transfected cells. As such, targeting apoptosis as a means of enhancing transfection efficiency seems to be a viable option, provided that the cell line involved presents toxicity as a chokepoint. Recently, Kim *et al.* (2002) reported that the co-transfection of anti-apoptotic genes, bcl-2 and bcl-xL, with the reporter plasmid resulted in a significantly enhanced transfection efficiency in neural stem cells. Furthermore, the authors

supplemented the cells with B27 supplement, which has been shown to have a pro-survival effect on neural stem cells (Svendsen *et al.*, 1995).

Mechanistic Factors Affecting Transfection efficiency

Other factors that affect transfection efficiency are transfection-method specific. Often, based on the understanding of the transfection mechanism, transfection efficiency can be enhanced by targeting the method specific chokepoint. For example, the incorporation of DNA compacting peptides (short peptidal sequence KTPKKAKKP) during lipofection has been shown to enhance the serum stability of DNA lipoplexes, which is a major problem associated with liposome-mediated transfection (Schwartz *et al.*, 1999). Furthermore, the addition of a "glycerol shock" step during calcium phosphate mediated transfection has been shown to destabilize endosomal compartments, leading to the early release of transfected DNA from the endosomal-lysosomal compartments and enhancing transfection efficiency in certain cell lines (Lopata *et al.*, 1984). However, most of these transfection enhancers are based on a clear understanding of the mechanism, which may not always be available. Moreover, enhancement effects are generally associated with the specific transfection method, which makes them less than readily translatable to other techniques (Haberland *et al.*, 1999). Worse yet, certain transfection enhancers seem to only be able to enhance transfection efficiency in certain cell lines, making the general use of these enhancers ineffective (Ohmiya *et al.*, 2002).

Transfection by Chemical Means

As briefly described in previous sections, transfection can be achieved by a variety of chemical means such as calcium phosphate, lipoplexes, and polyplexes.

Optimizable Parameters of Calcium Phosphate Precipitation

As stated earlier, calcium phosphate precipitation (CaPi) is based upon the formation of insoluble calcium phosphate aggregates complexed with plasmid DNA. The basic features of this procedure include mixture of DNA with $CaCl_2$ and sodium phosphate in buffered saline in a controlled manner, incubation at room temperature during which formation of calcium phosphate-DNA complexes occurs, and dispersion of precipitates onto cultured cells. This CaPi-DNA complex is allowed to settle (mostly by gravity but some protocols include a centrifugation step) upon the surface of the cells, and either phagocytosis or endocytosis occurs. After removal of the DNA-containing medium, a brief incubation with dimethylsulfoxide or glycerol is occasionally employed to enhance DNA uptake (Wilson *et al.*, 1995). The transfected DNA is processed through endosomal/lysosomal compartments and eventually arrives at the nucleus, where transcription occurs. Unlike DEAE-dextran, which was developed by Vaheri and Pagano (1965) near the same time frame, the CaPi technique can be employed to generate both transient and stable transfectants. In recent years, several factors have been shown to be critical to form the optimal hydroxyapatite for maximal transfection efficiency.

pH of Buffered Sodium Phosphate Solution

DNA-calcium phosphate co-precipitates arise spontaneously in supersaturated solutions. However, the quality of the precipitates can have severe implications on the outcome of transfection, with small precipitate particles between 1-3μm being optimal for maximal transfection efficiency (Jiang *et al.*, 2004). Particles that are too large are presumably not efficiently endocytosed, while particles too small may not be sufficient in triggering an endocytic response (Jiang *et al.*, 2004).

In order to generate particles of the optimal size, the pH of the phosphate buffered saline solution with which the DNA-$CaCl_2$ solution is mixed is optimized as it can determine the kinetics of the precipitate formation, a critical aspect of forming precipitates of the optimal size (Wilson *et al.*, 1995 and Jordan *et al.*, 1996). Furthermore, transmission electron microscopy data revealed that crystallites formed in pH less than 7.01 were more particulate in shape, a clear distinction from the crystallites formed in pH above 7.12, where the clusters appeared more irregular in appearance (Yang and Yang, 1997). According to Wilson (1995), the pH of the solutions that yield maximal transfection efficiency for bovine chromaffin cells is 6.95. The formation of precipitate was observed to correspond to an increase in turbidity of the mixed solution at A_{660} during the incubation period at room temperature. Although spectrophotometric data can be of use to guide an inexperienced user, the increase in turbidity does not account for the quality of the precipitate, which is a far more critical element than the mere formation of the precipitate accounted for by the increase in turbidity. Moreover, the pH of the solution is incrementally lower that the accepted value of 7.1, as suggested by Promega Corporation (Promega transfection guide). It is more than likely that during Wilson's protocol (1995), the longer reported incubation at room temperature (30-40 minutes vs. 20 minutes) compensated for the slightly lowered pH used in his solutions.

Concentration of DNA

Even as far back as the original publication by Graham and van der Eb (1973), the concentration of transfected DNA was deemed to be one of the factors affecting transfection efficiency via the calcium phosphate method. Chen and Okayama (1987) observed that the number of stable transformants varied with the amount of transfected DNA. In certain reports, increasing amounts of exogenous DNA resulted in enhancing transfection efficiency

(Reeves *et al.*, 1985). However, Chen and Okayama reported that the optimal concentration of DNA resulted in the transition of coarse to fine precipitates when complexed with calcium phosphate that coincided with maximum transfection efficiency. To that end, Jordan *et al.*, (1996) verified the observation and attributed the DNA concentration effect on transfection efficiency by altering precipitate formation kinetics. At extremely high concentrations of DNA (>50μg/mL DNA), precipitate formation was inhibited and less than 20% of the transfected DNA was associated with a precipitate after a 20-minute incubation period. Aside from precipitate kinetics, optimal concentrations of transfected DNA also varied between cell lines as was observed by Ding and Tan (1989). Thus, optimizing DNA concentration during transfection remains one of the empirical parameters to be determined experimentally.

Length of Precipitate Formation

After the addition of $CaCl_2$-DNA solution to the buffered phosphate solution, the mixture is incubated at room temperature for a period of time ranging from 5 to 30 minutes (O'Mahoney and Adams, 1994 and Seelos, 1997). The duration of incubation was determined to be one of the critical parameters, in addition to pH of the buffered phosphate solution and DNA concentration. In 1996, a shocking report by Jordan *et al.* reported in *Nucleic Acids Research* that the standard incubation period and the slow mixture of the DNA-$CaCl_2$ with the buffered sodium phosphate solution yielded precipitates that were far from optimal in terms of transfection efficiency. In terms of incubation, Jordan found that by mixing the solutions quickly, soluble DNA in the reaction mix is bound to an insoluble complex with calcium phosphate in less than 1 minute. Furthermore, the authors went on to further explain that by extending the reaction time to 20 minutes, aggregation and/or growth of particles result in a reduction in the level of transgene expression, presumably by the

inadequate endocytosis of the larger DNA-CaPi particles (Jordan *et al.*, 1996). This was the first time that the kinetics of CaPi precipitation were linked to transfection efficiency. As explained by the authors, after the initial addition of DNA-$CaCl_2$ solution to the buffered sodium phosphate, precipitates consisted of a large number of very small particles covering the surface of individual cells almost completely. After 40 minutes of incubation, the precipitates have aggregated to form fewer but larger particles, some even as big as the cells themselves (Jordan *et al.*, 1996). Thus, the new protocol, along with the detection assay also described in the report, has gained favor due to the comprehensive nature of the study.

However, the report by Jordan *et al.* (1996) was rebuffed by several sources, indicating that the quick mixing had a severe impact on the formation of precipitates. Christian Seelos (1997) compared the standard mixing method (dropwise addition of DNA-$CaCl_2$ to buffered sodium phosphate) and incubation period (10-30 minutes) with the Jordan protocol and discovered that while transfection efficiency was reduced during longer periods of incubation with the Jordan protocol, the standard method actually yielded higher transgene expression after a 30-minute incubation. While Seelos carefully qualified his statement to pertain only to rat embryo cells (RECs), this nonetheless pointed out that the method of addition could potentially affect the kinetics of the precipitate formation, resulting in enhancement or suppression of transfection efficiency. Recently, Chowdhury *et al.* (2004) reported the inclusion of magnesium to affect the kinetics of precipitate formation, resulting in the transition of particle diameter from 2.5μm to 500nm, enhancing transgene expression at least 40 times.

Length of Transfection

Another of the variables originally examined by Graham and van der Eb (1973) was the length of time on which DNA precipitates were left incubating with the cells. Since then,

the optimal length of transfection to yield the highest transfection efficiency has been highly variable (Table 2). As is recommended by the Promega Transfection Guide, the usual length of transfection falls between 4-16 hours. However, as little as one hour (Jiang *et al.*, 2004) or as long as 20 hours (Hilliard *et al.*, 1996) have been reported as optimal durations for transfection. The length of time is primarily dependent on cellular tolerance of DNA-$CaPO_4$ precipitates. While a longer duration of transfection will likely lead to higher transfection efficiency (Hilliard *et al.*, 1996), it will also lead to higher toxicity in a cell-type dependent manner (Jiang *et al.*, 2004). Thus, duration of transfection is also a variable that requires empirical determination, on a cell-type to cell-type basis.

Table 2: Range of Conditions for Calcium Phosphate Transfection

		Reference
DNA concentration*	10 – 20μg DNA/mL <25μg DNA/mL transfection mixture	Schenborn and Goiffon, 2000 Jordan *et al.*, 1996
Calcium concentration	2.5M stock / 12.5mM final	
pH of Buffered phosphate solution	BES pH 6.95 HBS pH 7.05	Chen and Okayama, 1987 Jordan *et al.*, 1996
Length of precipitate formation	1 minute to 30 minutes	Chen and Okayama, 1987 Jordan *et al.*, 1996 O'Mahoney and Adams, 1994 Seelos, 1997
Length of transfection*	1-3 hours 4-16 hours 4 hour 4-5 hours 6 hours 8 hours	Jiang *et al.*, 2004 Promega transfection guide Batard *et al.*, 2001 Segura *et al.*, 2001 Urabe *et al.*, 2000 Gaunitz *et al.*, 1996

* to be determined empirically for each cell line

Lipoplexes

Liposome-mediated gene transfer, collectively termed "lipoplexes", has been extensively studied to become the prominent gene transfer vector for non-viral gene transfer. While exhibiting higher transfection efficiency than most other non-viral vectors, the lack of specialized equipment makes this technique especially attractive. Recently, the elucidation

of several mechanistic aspects during lipoplex-mediated transfection has added crucial pieces to the understanding of the underlying mechanism.

Formation of liposome

Generally, the polycationic charges from the structural elements of lipoplexes provide a means for electrostatic interaction with the DNA phosphate, resulting in compaction of the nucleic acid by and complexation with the amphiphile, thereby causing its shielding from attack by exogenous and endogenous DNase (Oberle *et al.,* 2000). The morphological appearance of these lipoplexes has been described as multilamellar aggregates (Gustafsson *et al.*, 1995), rod-shaped structures (Gershon *et al.*, 1993), DNA coating on the surface of cationic liposomes (Eastman *et al.*, 1997), or bead-on-string-like complexes (Sternberg *et al.*, 1994), with each of the different structures potentially affecting transfection efficiency.

Recently, Oberle has proposed a three-step mechanism during the formation of lipoplexes. In the first step, supercoiled plasmid DNA interacts electrostatically with a monolayer of the cationic amphiphiles. In a second step, bean-like structures arise. Given the relatively smooth surface of the lipoplexes and their dimensions, their appearance is interpreted to result from the coating of single supercoiled plasmids with a bilayer of cationic lipids, the charged amphiphile head groups facing the hydrophilic environment in which the complex is formed. Then, the unilamellar lipoplex increases in size from 30 to 50-70nm in width. Oberle concluded that the plasmid is surrounded by 3-5 bilayers of the amphiphile, based on atomic force microscopy. The eventual size of the transfecting complex is governed by fusion events between individually wrapped amphiphile/DNA complexes.

Cationic lipoplexes, with their positively charged outer surfaces, are electrostatically attracted to the membrane surface of the cell. DNA complexes then gain access across the cell membrane by endocytosis, and are processed through the endosome-lysosomal

pathways. Lipoplexes have the ability to aid early release from the endosome-lysosomal compartments, but the DNA must be released from the liposomes before it can gain access to the nucleus.

Release of DNA

Zabner *et al.* (1995) showed that DNA-lipoplexes that are microinjected directly into the nucleus do not induce high expression levels. This demonstrated the requirement for plasmid DNA to escape its association with liposomes before entering the nucleus, thereby leading transgene expression. In 1996, Xu and Szoka proposed the following mechanism for the release of DNA during transfection. First, the cell surface associated complex is internalized into an endosome. The complex initiates a destabilization of the endosome membrane that results in flip-flop of anionic lipids that are predominately located on the cytoplasmic face of the membrane. The anionic lipids laterally diffuse into the complex and form charge-neutralized ion pairs with the cationic lipids. This displaces the plasmid DNA from the complex and permits DNA entry into the cytoplasm (Xu and Szoka, 1996). The DNA then gains access to the nucleus either during post-mitotic nuclear membrane dissolution or through active transport via the nuclear pore complex, much akin to that of calcium phosphate transfection.

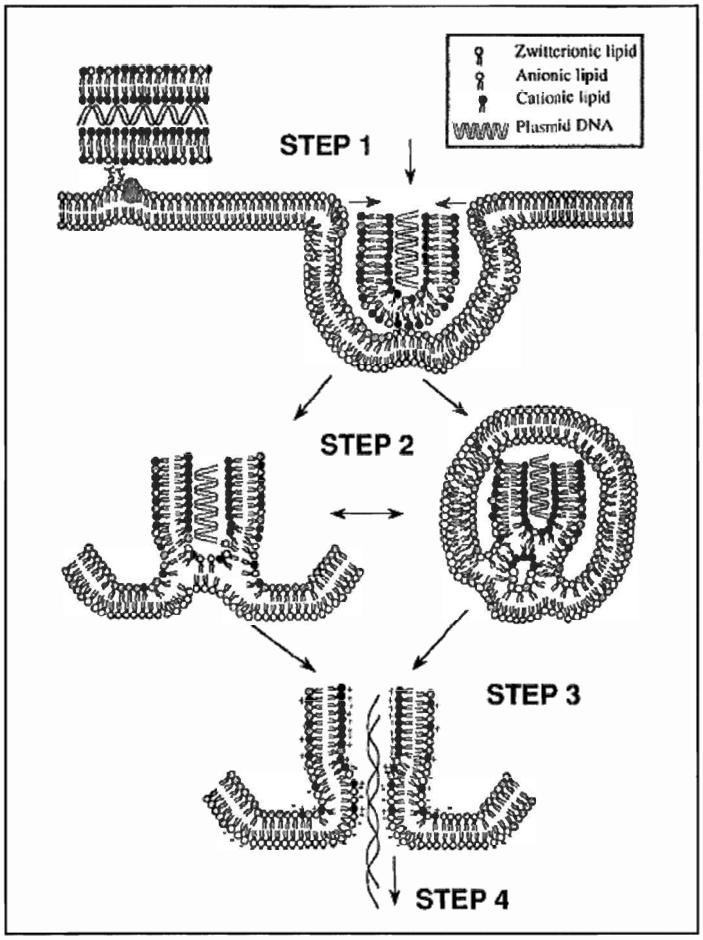


Figure 2: Mechanism of release of plasmid DNA from lipoplexes. Step 1. After electrostatic interaction with the cell membrane, cationic liposome/DNA complexes are endocytosed. Step 2. In the early endosome, membrane destabilization results in anionic phospholipid flip-flop. Step 3. The anionic lipids diffuse into the complex and form a charge neutral ion pair with cationic lipids. Step 4. The DNA dissociates from the complex and is released into the cytoplasm (Reproduced from Xu and Szoka, 1996).

Optimizable parameters

Because of the diverse structure of amphiphiles that form lipoplexes with DNA, variables that are optimizable have been greatly simplified, as compared to that of calcium phosphate. While the general parameters such as DNA concentration and duration of

transfection remain to be determined empirically, there are certain parameters that are lipoplex specific. These include structure of lipids, charge ratio and lipoplex size.

Structure of lipids

Unlike calcium phosphate mediated transfection, methods for increasing transfection efficiency using liposomes have generally gone the way of synthesizing new amphiphiles. Structurally, cationic lipids consist of a cationic moiety, a linker region and a fatty acid derivative or hydrophobic region (see Figure 3). The cationic moiety is primarily responsible for interaction with DNA, as well as forming electrostatic interactions with the plasma membrane.

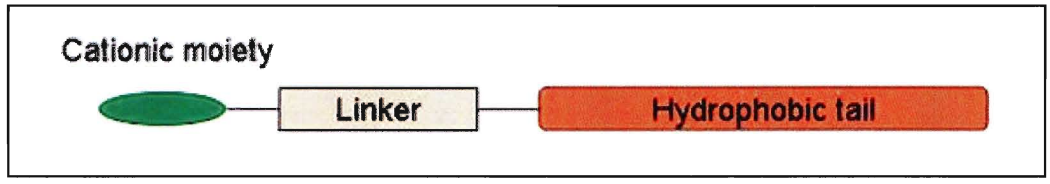


Figure 3: Structure of a cationic lipid, with a cationic moiety, linker and a hydrophobic tail. Figure adapted from Fichert et al., 2000.

The cationic moiety has numerous possibilities for modifications in terms of both numbers of positive charges and chemical structure. These can include quaternary ammonium (i.e. DOTAP), primary, secondary and tertiary amines, guanidinium, heterocyclics, amino acids and peptides. Insertion of cationic entities of moderate pKa such as imidazole is also of particular interest for complex liberation into the endosome. Cationic global charge increase at lower pH is believed to create a proton-pumping effect by counter ion accumulation, inducing endosome lysis.

There is also considerable variability in the hydrophobic aspects of cationic lipid. Generally speaking, the choice of lipids is predominantly between a two-hydrocarbon chain (i.e. DOTAP) and a cholesterol moiety (i.e. DC-chol).

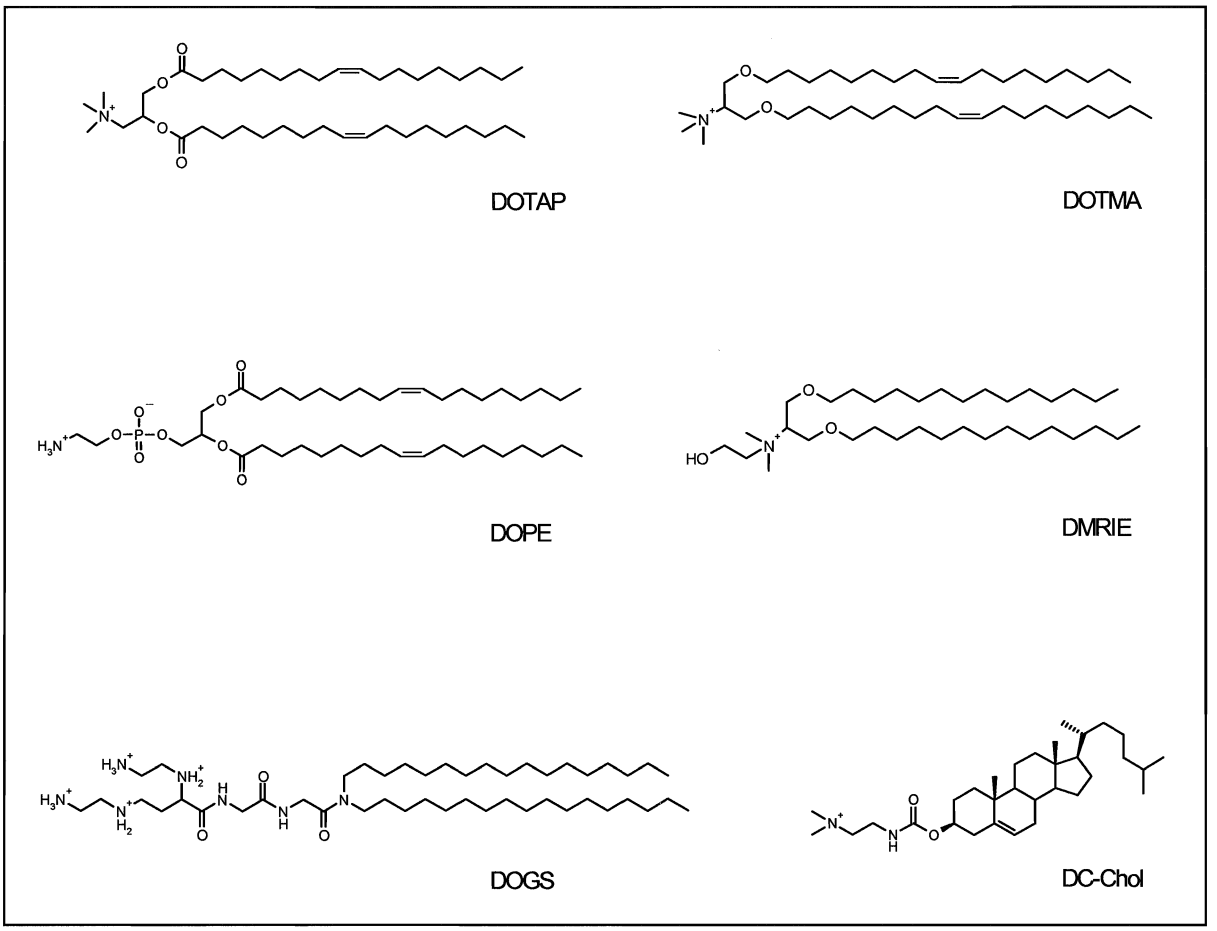


Figure 4. Structures of commonly utilized lipoplexes. Structures adapted from Zabner, 1997

Furthermore, chain lengths from C8:0 to C18:1 (C<Chain length>:<degrees of saturation>) and degrees of saturation are also highly variable between cationic lipids (Tranchant *et al.*, 2004). As there is considerable information on the structural/activity relationship between the variable lipid structures and transfection efficiency, various synthetic lipids are continually being developed (Ferrari *et al.*, 2002). In terms of chain

length, shorter hydrocarbon chains seem to be better for *in-vitro* transfection efficiency while longer chains allow for better transfection *in-vivo* (Floch *et al.,* 2000). Furthermore, mono-unsaturated fatty acid chains yield better transfection results, possibly through modulation of membrane fluidity, but long-term storage is more problematic since double-bonds are more prone to oxidation than their alkyl counterparts.

Lipid/DNA Ratio

Another important parameter that is usually empirically determined is the ratio of DNA to lipid. In essence, the ratio of DNA to lipid can significantly affect two physiochemical characteristics of lipoplexes: zeta potential and lipoplex size.

Zeta potential refers to the bulk charge of the resulting lipoplex. In the case of cationic lipids, a higher ratio of lipid to DNA will generally result in a more positive zeta potential (Sakurai *et al.*, 2000). A bulk positive charge is critical for electrostatic interaction of the DNA-lipoplex with the negatively charged cellular membrane. However, merely increasing the lipid/DNA ratio is not necessarily the path towards enhanced transfection efficiency as higher concentrations of lipids have been associated with elevated toxicity. To address this problem, newer polycationic formulations such as droctadecyl-amidoglycylspermine (DOGS) have incorporated multicationic head groups such as spermine so that lower amounts of lipid are needed to yield a positive zeta potential.

Aside from zeta potential, lipoplex size is largely affected by lipid structure and lipid/DNA ratio. While some reports have argued that size is not associated with transfection efficiency (Stegmann and Legendre, 1997), recent reports have concluded that larger lipoplexes are more efficient, at least in *in-vitro* studies (Almofti *et al.*, 2003). Furthermore, Ross and Hui (1999) determined that lipoplex size affects whether serum-induced inhibition of transfection becomes a factor in determining the outcome of transfection.

Helper Lipids/Other Enhancing Agents

Generally speaking, most cationic lipid mediated transfection involves the use of an anionic, helper lipid to enhance transfection efficiency. The commonly used helper lipids include cholesterol, dioleoyl phosphatidylcholine (DOPC) and dioleoyl phosphatidyl-ethanolamine (DOPE). These lipids presumably have some endosomal destabilizing activity, which allows for the early release of DNA into the cytosol from endosomal compartments (Farhood *et al.*, 1995). Aside from endosomal destabilizing agents, numerous enhancing agents have been investigated to enhance liposome-mediate transfection (see Table 3). These agents include nuclease inhibitors (Ni *et al.*, 1999), mitogens (Rippe *et al.*, 1990, Yanagihara *et al.*, 2000 and Ohmiya *et al.*, 2002), DNA complexation agents (Kariko *et al.*, 1998 and Schwartz *et al.*, 1999), membrane permeants (Lawrie *et al.*, 1999, Fong *et al.*, 2004, Freeman and Niven, 1996), lysosomotropics (Ciftci and Levy, 2001 and Hasan *et al.*, 1991), sedimentation enhancers (Krotz *et al.*, 2003 and Huth *et al.*, 2004), membrane fusion enhancers (Okimoto *et al.*, 2001 and Haberland *et al.*, 1999), nuclear translocation enhancers (Chan *et al.*, 2000) and receptor targeting molecules (Yanagihara and Chang, 1999).

Polyplexes

One of the earliest agents used for non-viral gene transfer, DEAE-dextran (Vaheri and Pagano, 1965) is a polycationic polymer based DNA-complexing agent. Structurally, polyamines carrying a positive charge bind the negatively charged phosphate backbone, condensing it into compact, ordered particles of 20-200 nm in diameter (Liu *et al.*, 2001). At a critical ratio of polycation to DNA, the aggregation leads to a localized bending or distortion, which facilitates the formation of rods, toroids (Golan *et al.*, 1999) and spheroids (Liu *et al.*, 2001). The size of the aggregates is an important element in determining

transfection efficiency, as PEI that condense DNA into compact particles (80-100nm vs. 2μm) have been shown to be much more efficient in terms of transfection efficiency (Petersen *et al.*, 2002). Aside from DNA compaction, binding of the polycations to DNA also leads to charge neutralization or even slightly positive aggregates, which have been shown to interact with the negatively charged proteoglycans of the cell membrane (Erbacher *et al.*, 1999). These aggregates, in turn, trigger endocytosis and internalization of the DNA-polyplex aggregates.

Before DNA can be expressed, it must efficiently escape endosomal/lysosomal destruction. The most common hypothesis is the mechanism by which polyplexes aid in the early release of transfected DNA from the endosome/lysosome into the cytosol. The "proton sponge" mechanism refers to the ability for the polycationic polyplex to buffer the pH change in the endosomal/lysosomal compartments. The buffering of pH leads to a wide variety of consequences. In the endosomal compartment, a slew of acidic proteases are activated by a pH change. The increase in protease activity has a putative role in the processing and maturation of lysosomal nucleases (Coonrod *et al.,* 1997). Upon endosome-lysosome fusion, the proton sponge mechanism inhibits acidic nucleases, namely DNase II, by buffering a pH change, and thus, enhancing the stability of exogenous DNA (Akinc and Langer, 2002). Although this mechanism has been widely accepted, it has recently been called into question from studies of PEI (Gebhart and Kabanov, 2001). It was reported that the buffering capacity of PEIs is negligibly low between pH 4.5 to 7.4, which represents the range of pHs within the vesicular compartments during the transition from early to late endosomal compartments and then to the lysosomal compartments. Alternatively, another mechanism suggested that the polycations attract counter-ions once internalized into an endosome. As charge neutralization occurs, the increase in solute concentration leads to an influx of water, leading to the rupture of the endosome. Consequently, DNA is released into the cytosol.

Cytosolic DNA-polyplexes afford some protection against cytosolic DNases. As the complex traverses the nuclear membrane, disassociation of the vector from the DNA has also been shown to be a barrier to transfection (Schaffer *et al.*, 2000).

Polymer Structure

Much like lipoplexes, the structural diversity of polymeric transfection agents makes polyplexes especially attractive for systematic studies. Although grouped into a singular category, polycationic polyplexes can differ both in chemical structure and the number of repeating units, as well as the architecture of the polymer backbone. For instance, the polymer backbone can be linear, randomly branched, dendrimeric, block-or-graft copolymer. As a result, different polyplexes are systematically synthesized and evaluated in terms of efficiency, toxicity and bioavailability (Ledley, 1995).

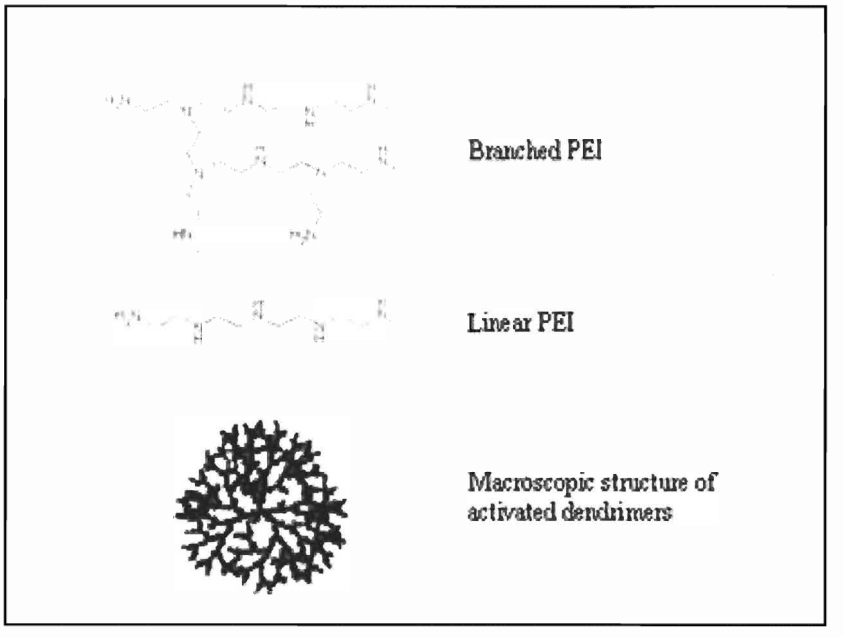


Figure 5. Structures of commonly used polyplex agents. Generally, polymers are activated from their monomeric counterpart to induce polymerization. Their macroscopic structures are commonly extremely complex. Structures adapted from Thomas and Klibanov, 2003.

N to P Ratio

Similar to zeta potential, N to P ratio refers to molar ratio of the nitrogen-containing groups to the phosphate containing backbone. The N/P ratio reflects the overall positive to negative charge balance of the DNA-polyplex complex. Polyplexes formed at high N/P ratios are more positively charged; therefore, they are likely to interact more effectively with the negatively charged cell surface via nonspecific charge interaction. Generally, transfection efficiency increases with an increase in N/P ratio owing to the fact that an increase in the positive charge of the corresponding polyplex and increase in polyplex content would lead to elevated cellular uptake and more efficient endosome release of the internalized polyplexes (Guo and Lee, 1999). Again, this is one of the variables that are empirically determined and optimized based on each polyplex transfection reagent.

Transfection Enhancing Agents

Aside from developing newer and more efficient DNA delivery vectors, there has also been the development of various transfection-enhancing agents. These agents are diverse in their nature and can include peptides, fatty acid derivatives, DNA-binding dyes, hormones, various chemicals, nucleic acids and ultrasound (see Table 3 for a complete list). Mechanistically, they act upon various chokepoints during the transfection process, namely to overcome the three major hurdles: (1) the cell membrane, (2) nucleases and (3) nuclear membrane.

Membrane Fusion Enhancers

As one would expect, an enhancement in membrane fusion should also increase transfection efficiency. Haberland *et al.* (1999) demonstrated that the use of calcium ions

during histone H1 mediated transfection enhanced transfection efficiency in ECV 304 cells. The authors concluded that the free calcium ions resulted in the formation of CaPi microprecipitates, which are known to have fusogenic and membranolytic activity. Presumably, the additional precipitates not only aid in traversing the cellular membrane but also aid to destabilize endosomes, resulting in the early release of DNA into the cytosol. However, such enhancement was not observed with liposome-mediated transfection.

Receptor Targeting Molecule

As a method of enhancing endocytosis, additional receptor targeting ligands have been added to lipoplex-mediated transfection. Transferrin has been used to supplement cationic liposomes and has been shown to enhance transfection efficiency in HeLa and human tracheal epithelial cells (Cheng, 1996). Yanagihara and Cheng (1999) show that lectins can also enhance transfection efficiency in lung carcinoma cells lines A549, Calu3 and H292 by associating with receptor-associated glycans. Aside from enhancing endocytosis via a receptor-mediated pathway, the use of specific ligands can also be utilized for tissue specific targeting.

Sedimentation Enhancers

During the transfection process, liquid phase diffusion is often a major limiting factor. As DNA complexes remain suspended in the media, they will remain unavailable for endocytosis and internalization. Several reports have indicated an enhancement in transfection efficiency by physically concentrating the DNA-complex onto the surface of the adherent cells. Luo and Saltzman (2000a) demonstrated an 8.5 fold increase in transfection efficiency using silica nanoparticles-polyplex as a DNA delivery vector to CHO cells by

incorporating a centrifugation step to enhance the "effective" concentration of DNA onto the cell surface. Moreover, the incorporation of magnetofectin and use of a magnetic field to "drive" DNA-nanoparticles onto adherent cells have been recently termed "magnetofection" (Krötz *et al.*, 2003 and Krötz *et al.*, 2003b). The enhancement of transfection efficiency was reported to be up to 360-fold relative to their non-magnetofectin counterpart. The enhancement was demonstrated for both lipofection (using FuGENE, Lipofectamine and DMRIE) and polyplexes (PEI).

DNA-Complexing Agent

As stated earlier, one of the difficulties of employing liposomes as a gene delivery vector is mainly their instability in the presence of serum. By employing short cationic peptides derived from histone sequences, Schwartz *et al.* (1999) were able to reduce the serum inhibition of transfection efficiency using lipospermine DOGS. In the presence of peptide H9-2, transfection efficiency was approximately two orders of magnitude higher in 20% FCS using lipofection reagent RPR 120535. Furthermore, employing these peptidal sequences allowed for a reduction in the amount of lipofection reagent used and thus, reduced its toxicity. The authors went on to conclude that the primary role for these peptides served to compact DNA, leading to a modification of the intracellular fate of the DNA particles when performed in the presence of serum

Membrane Permeant

A number of cationic, membrane-permeant small molecules can rapidly partition through all membranes of living cells to enter the nucleus. They then bind with high affinity to nuclear DNA (Fong *et al.*, 2004). Fong *et al.* (2004) used DNA-binding dye Hoechst

33258 and saw an increase in transfection efficiency for both liposome and PEI mediated transfection. Hoechst 33258 acted as a membrane permeant, leading to rapid transition between all membrane barriers including plasma membrane, endosome and nuclear membrane. Thus, the ability for these membrane-permeant dyes to transfect non-dividing cells came as no surprise. This was particularly attractive as it has been a long known problem that lipofection required rapidly dividing cells for transfection to occur. When Hoechst 33258 was used in conjunction with liposomes, transfection efficiency was as high as 80% for B16-F10 cells (Fong *et al.*, 2004).

Lysosomotropics

Endosomal and lysosomal compartments bear special significance to efficient transfection. Aside from the hostile pH environment during the acidification transitioning from early to late endosome and then to the lysosome, a slew of acidic nucleases is present to degrade exogenous DNA. Chloroquine, glycerol and DMSO are known lysosomotropics that induce the influx of water into endosomal/lysosomal compartments, causing their rupture and release of transfected DNA into the cytosol. More recently, Ciftci and Levy (2001) reported the use of sucrose as a lysosomotropic agent in conjunction with Lipofectamine. The presumptive mechanism is related to the inability of cells to degrade sucrose, due to the absence of disaccharide enzymes (Kato *et al.*, 1984). The resulting increase in osmotic pressure within the endosomal/lysosomal compartments led to the disruption of the endosome. Sucrose was found to be a more effective and less toxic transfection enhancer than chloroquine and polyvinylpyrolidone on COS, 293 and CHO cells.

Nuclease Inhibitor

A variety of agents can complex DNA and protect it from nuclease degradation. These peptidal transfection-enhancing agents bind DNA and change its conformation so that it becomes less accessible to nuclease digestion. Reagents that demonstrate a known inhibitory role to nucleases include: chitosan (Bozkir and Saka, 2004), protamine (Ni *et al.,* 1999), DMI-2 (Ross *et al.,* 1998), ATA (Walther *et al.,* 2004), poly (D, L-lactide-co-glycolide) microspheres (Gebrekidan *et al.,* 2000), Chol-Q (Kisoon *et al.,* 2002) and pTMAEM (Su *et al.,* 2002).

Mitogens

One of the criteria for successful transfection via non-viral means is cell proliferation (see Section Cell Proliferation/Cell Density). To enhance cell proliferation, mitogens such as insulin were used to enhance transfection efficiency. Ohmiya *et al.* (2002) demonstrated that insulin enhanced the proportion of cells in the S and G2M phase of the cell cycle, resulting in an increase in transfection efficiency via the nuclear membrane dissolution hypothesis. Ohmiya *et al.* showed an enhancement of transfection efficiency by insulin in MKN1 and HT29 cells. However, BxPC3 cells did not yield a significant difference, indicating the cell-type dependence that is often observed with protocols/enhancement agents that are designed to increase transfection efficiency.

For a full list of transfection enhancing agents, refer to Table 3.

Table 3: Agents used to Enhance Transfection Efficiency

Transfection Enhancer	Mech. of Action	Method of Transfection	Cells involved	Reference
Albumin microbubbles + USE	D		293	Frenkel *et al.*, 2002
Bcl-2, Bcl-xL and B27	H	Lipofection	PGC	Watanabe *et al.*, 1997 Kim *et al.*, 2002
Calcium	I	Peptide mediated	ECV-304 HepG2	Haberland *et al.*, 1999
CalPhos Maximizer	U/K	Calcium Phosphate	CHO-K1 BHK-21 293 HeLa	Zhang and Kain, 1996
Carrier DNA	E	Calcium Phosphate	CV-1 NIH3T3 CHO	Strain and Wyllie, 1984 Strain *et al.*, 1985 Shore and Reddy, 1989 Nickoloff and Reynolds, 1992
Chloroquine	E	Calcium Phosphate	3T6 Fischer Rat-1	Luthman and Magnusson, 1983 Hasan *et al.*, 1991
Cortisol	J	Calcium Phosphate, Polymer	3Y1 HeLa	Bernasconi *et al.*, 1997
DMSO shock	D	Calcium Phosphate	L cells	Lopata *et al.*, 1984
Gelatin + USE	C, D		RGM-1	Hosseinkhani *et al.*, 2002
Glycerol shock	D	Calcium Phosphate	L-cells MCF-10	Lopata *et al.*, 1984 Basolo *et al.*, 1990
Hoechst 33258	D	Lipofection, Polymer	B16-F10	Fong *et al.*, 2004
Insulin	B	Lipofection	MKN1, HT29	Rippe *et al.*, 1990 Yanagihara *et al.*, 2000 Ohmiya *et al.*, 2002
KTPKKAKKP	C	Lipofection	3T3, 3LL, H460, H1299	Schwartz *et al.*, 1999
Lectin (GSI, MAA)	K	Lipofection	A549, Calu3, H292	Yanagihara and Cheng, 1999
Magnesium	H	Calcium Phosphate	HeLa, 3T3	Chowdhury *et al.*, 2004

Transfection Enhancer	Mech. of Action	Method of Transfection	Cells involved	Reference
Magnetofectin	F	Lipofection	HeLa, BEAS-2B, HEP-G2, HUVEC, endothelials	Krotz *et al.*, 2003 Krotz *et al.*, 2003b Huth *et al.*, 2004
NH_4Cl, FCCP and 3-MA	E	Calcium Phosphate	HeLa	Ege *et al.*, 1984
NLS	J		HTC	Chan *et al.*, 2000
PAMAM	I	Lipofection	numerous	Kukowska-Latallo *et al.*, 1996 Roessler *et al.*, 2001
Phosphate	C, H	Lipofection	HOS	Kariko *et al.*, 1998
PLGA-PEG-PLGA	**		293	Jeong *et al.*, 2004
Polybrene	C	Lipofection	BHK, 208F	Abe *et al.*, 1998
Protamine	A	Lipofection	Huh7	Ni *et al.*, 1999
SAINT-2	D	Lipofection	COS-7 CV-1	Van der Wonde *et al.*, 1997
Sodium Butyrate			CV-1 3T3	Gorman *et al.*, 1983
Sodium Glycolate	D	Lipofection	COS-7	Freeman and Niven, 1996
Sucrose	E	Lipofection	COS, 293, CHO	Ciftci and Levy, 2001
TPA	*	Calcium Phosphate	3T3	Reston *et al.*, 1991 Reston *et al.*, 1993
Transferrin	K	Lipofection	HeLa, A546, Calu3, H292	Cheng, 1996 Yanagihara *et al.*, 2000
Ultrasound	D	Lipofection	VSMC, EC	Lawrie *et al.*, 1999
VSV G-protein	I	Calcium Phosphate, Lipofection	BHK, 208F	Okimoto *et al.*, 2001

The mechanism of transfection enhancement is extracted from the original publication. As some are proposed mechanisms, as suggested by the authors of the publication, new mechanistic insights may change these categories. A-Nuclease inhibitor. B-Mitogens. C-DNA complexing agents. D-Membrane permeants. E-Lysosomotropics. F-Sedimentation enhancers. H-Miscellaneous. I-Membrane fusion enhancers. J-Nuclear translocation enhancer. K-Receptor targeting molecule. U/K-Mechanism unknown.

Aim of Current Study

Calcium phosphate-DNA co-precipitation technique, despite its low transfection efficiency, has been one of the most utilized methods of transfection because of the lack of specialized equipment, low cost and ease of finding the requisite components. Using this common transfection method, we examined the cellular and/or mechanistic factors that affect transfection efficiency in a cell-type dependent manner. This stemmed from the observation that optimized protocols have been shown to be efficient in certain cell lines, and not others. Worse yet, transfection-enhancing agents such as glycerol and DMSO seem to also affect transfection efficiency in a cell-type dependent manner. Thus, using various commonly used cell lines (HEK-293, HeLa and MDBK), an examination of these factors was undertaken, in the hopes of identifying factors that will enhance transfection efficiency.

Chapter II

Materials and Methods

Bacteria, Media, Growth and Storage

Several strains of *Escherichia coli* K12 were used as hosts in various cloning experiments described in this report. These include **DH5αF'** (F'*/endA1 hsdR17*($r_K^- m_K^+$) *glnV44 thi-1 recA1 gyrA*(Nal^r) *relA1* Δ(*laclZYA-argF)U169 deoR*(ϕ80*dlacΔ(lacZ)M15*); (NEB Catalogue 2002-2003) and **HB101** (F' *Δ(gpt-proA)62 leuB6 glnV44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL20(Str^r) xyl-5 mtl-1 recA13)*. These cells were typically grown in LB broth (per liter: 10g bacto-tryptone, 5g yeast extract, 10g NaCl, pH 7.0 – 7.2) at 37°C with vigorous shaking.

Bacterial cells in liquid medium were kept at 4°C for temporary storage, or in 20% glycerol at –70°C for extended storage as stocks. To recover bacterial cultures from frozen stocks, a small volume of a growth medium was inoculated with a loopful of frozen cells.

Plasmids

All plasmids that were used in assays and as sources of restriction fragments for cloning purposes were obtained from laboratory stocks. In instances where laboratory stock did not contain the required plasmid, it was obtained from commercial sources. Regardless,

all plasmids used in this thesis have been saved in the laboratory stocks and can be made available upon request. Plasmid restriction maps are available from manufacturer's web sites; other plasmids that were constructed are described in detail in the RESULTS section and APPENDIX.

Enzymes

Restriction Enzymes

The restriction enzymes used in this study were obtained from commercial suppliers, namely New England Biolabs (NEB) and MBI Fermentas. All enzymes were received in solutions of various storage buffers containing 50% glycerol. The reaction mixture was usually in a 20μL volume when the digest was used for analysis in gel electrophoresis or in larger volumes for preparative purposes. Digestions using crude DNA extract, as opposed to CsCl/ethidium bromide-banded preparations, required the use of a two-fold excess of enzyme and/or longer incubation time. Thus, in the digestion of DNA prepared by the alkaline-SDS method from a 1.5mL bacterial culture, about $1/50^{th}$ of total DNA yield was digested with 1 unit (usually defined as the amount needed to digest 1μg of DNA at 37°C in 1 hour) and incubated at 37°C for 2 or more hours, or as determined by gel electrophoresis.

Multiple-Enzyme Digests

On occasion, construction of some plasmids required fragments generated from simultaneous digestion with two or more different enzymes. Where appropriate, the use of a single enzyme buffer was particularly convenient in such cases even if the activity of the

enzymes being used was less than optimal in the universal buffer. Again, the variation in the activities of the enzymes was often not critical in carrying out many simultaneous digests, so that an equal amount of enzymes was usually sufficient to obtain complete digests. However, when the problem occasionally arose, similar adjustments in amount of enzyme and incubation time were made. Gel electrophoresis was used to determine the extent of the digestion (Refer to section **Gel Electrophoresis – Agarose Gels**). In instances where multiple enzymes were not compatible (as indicated in NEB catalog), sequential digests were performed to avoid problems such as star activity.

Inactivation of Enzyme

In some instances where, for example, a cloning experiment required multiple steps, it was necessary to inactivate the enzymes used in previous steps prior to proceeding to the next. Most enzymes used in this project were labile when subjected to heat-treatment at 65°C for 15 minutes. Others required higher temperatures such as 80°C to effectively destroy the activity (NEB catalogue, 2002-2003), while still others required phenol/chloroform-extraction followed by ethanol-precipitation. The phenol/chloroform reagent was prepared by mixing 1 volume of redistilled phenol, 1 volume of chloroform, and 0.1% 8-hydroxyquinoline (w/v). This was fully saturated in TE buffer (pH 8.0) and stored in the dark at 4°C indefinitely.

Phenol chloroform extraction was performed by adding equal volumes of phenol-chloroform solution to the enzyme digestion. The aqueous layer was removed and twice the volume of 95% ethanol stored at -20°C was added. The resulting precipitate was centrifuged and dried either by vacuum or short incubation at 37°C. The fragments were resuspended in the appropriate volume of TE buffer (pH 7.5).

Partial Digestion with Multi-Cut Enzymes

On occasions, partial digestion of plasmid DNA with restriction enzymes that recognize multiple sites on the same plasmid had to be used where unique restriction sites were not available.

Using such enzymes to linearize a supercoiled plasmid was accomplished using the method of Parker *et al.,* (1977), which is based on the differential binding of ethidium bromide to supercoiled and linear plasmid DNA. In a typical reaction volume of 100μL, 20μg of supercoiled DNA, the normal concentration of the universal buffer, ethidium bromide at a final concentration of 2.6μg/mL, and 1 unit of enzyme were added together. The incubation time, in which approximately 10% linear DNA was generated, was empirically determined for each enzyme. For example, 10μL aliquots were removed from the incubation reaction every minute and the enzymatic reaction was stopped by adding 5μL of 6X gel loading buffer. Using gel electrophoresis, the extent of reaction was estimated by the concentration of the different forms of plasmid DNA. Keeping the fraction of linear form to low levels (at about 10%) was desirable since it reduced the levels of linear DNA that contained more than one cut.

Klenow Fragment of DNA Polymerase I

The large scale peptide of DNA polymerase I due to cleavage by subtilisin (Jacobson *et al.,*, 1974), commonly called the Klenow Fragment, was used to remove protruding single stranded regions at the termini of DNA fragments produced by some restriction enzymes. In the presence of deoxynucleotide triphosphates (collectively termed dNTPs which include dGTP, dATP, dTTP, dCTP; purchased from MBI Fermentas), termini with recessed 3' ends

were filled by the polymerase activity of the fragment. However, the enzyme also possesses a 3'-to-5' exonuclease activity which can be utilized to remove a 3'-overhang. This procedure was used primarily to prepare DNA fragments that have incompatible termini for ligation by T4 DNA ligase.

Treatments with Klenow DNA polymerase were typically carried out using the supplied buffer. A 10X concentrate of this buffer (500mM Tris-HCl (pH 8.0 at 25°C, 50mM $MgCl_2$, 10mM DTT) was used to dilute to the operating concentrations. The reaction mixture (20μL) also contained up to 4μg of DNA and 0.05mM each of the dNTPs. The reaction mixture was incubated at 37°C for 10 minutes and was subsequently heat inactivated by incubating at 70°C for 10 minutes. The unincorporated dNTPs were removed by using DNA select columns (Norgen Biotek Ltd). The solution was then used in subsequent steps of molecular cloning.

Alkaline Phosphatase

In some applications either for cloning, enzymatic removal of the free 5'-phosphate was necessary. In cloning, the procedure was used to prevent the self-ligation of vector DNA, thus promoting the ligation between the vector and the fragment, which was usually left with the 5'-phosphate attached. Occasionally, the DNA fragment was in a restriction enzyme reaction mixture whose buffer system was compatible with alkaline phosphatase, so that changing the buffer was not necessary.

Between 1 and 2 units of enzyme per 1μg DNA were used. Incubation was at 37°C for 30 minutes. The enzyme was destroyed by either a) heating the reaction to 65°C for 15 minutes (for shrimp alkaline phosphatase) or b) a single phenol/chloroform extraction followed by ethanol precipitation (for calf intestinal alkaline phosphatase).

T4 DNA Ligase

For cloning purposes DNA fragments were ligated together using T4 DNA ligase. The buffer used consisted of 40mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 10mM DTT, and 0.5mM ATP. This was obtained as a 10X concentrate and stored in small aliquots at –20°C. Each aliquot was thawed once and was used within a period of 2-3 weeks, after which the solution was discarded. As a rule, this enzyme was used in all ligation experiments since it catalyses the covalent linkage between DNA fragments with blunt or recessed ends. Reaction mixtures were incubated at 15°C for a period ranging from 4 hours to incubation overnight.

Restriction fragments used in molecular cloning were usually gel-purified to enhance the efficiency of isolation of the desired clone (Refer to **Batch Preparation of Plasmid DNA – Purification of DNA from Agarose Gels**). In a typical reaction of 20μL, two fragments were added in a 10:1 molar ratio of insert to vector ensuring successful ligation.

Synthetic Oligonucleotides

Synthetic oligonucleotides were obtained by one of two ways. They were either purchased from Sigma Genosys or synthesized at Norgen using Beckman Oligo 1000 DNA synthesizer. Depending on the scale of the oligonucleotides, they were all diluted to 15μM in DEPC treated water. The diluted stock solutions were aliquoted to avoid repeated freezing and thawing, and were stored at -70°C for long term storage, or at -20°C for short term uses.

Table 4: List of oligonucleotides used in this work

Name	Sequence
5' RACE outer primer	5' GCT GAT GGC GAT GAA TGA AGA CTG 3'
5' RACE inner primer	5' CGC GGA TCC GAA CAC TGC GTT TGC TGG CTT TGA TG 3'
5' GSOP	5' ACC ACA AAG GGC TCC CTG GAA AAC AC 3'
5' GSIP	5' CGG GAT TCG TCT CCA TCC TGA TAG TCA TGG TA 3'
5' GSOP II	5' TTC CAG GCC TTC TTG GGG ACG TA 3'
5' GSIP II	5' CGG AAT TCG CCA GCA TTG AAG TCA CCC ATG AAA ATG 3'
GSP primer for RT	5' CTC TTC TTG ACC GTG GT 3'
Anchor primer	5' GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA GGT T_{11}T 3'
3' RACE outer primer	5' GCG AGC ACA GAA TTA ATA CGA CT 3'
3' RACE inner primer	5' CGC GGA TCC GAA TTA ATA CGA CTC ACT ATA GG 3'
3' GSOP	5' CTA CCA TGA CTA TCA GGA TGG AGA C 3'
3' GSIP	5' GGA ATT CGT GTT TTC CAG GGA GCC CTT TGT GGT 3'
Primer 1014-1033	5' CTG ACA TCC AGG GCC TCC TC 3'
psiRNA S4 sense oligo	5'-GAT CCG TTT CAT TTT CAT GGG TGA C TT CAA GAG AGT CAC CCA TGA AAA TGA AAT TTT TTG GAA A-3'
psiRNA S4 antisense oligo	5'-AGC TTT TCC AAA AAA TTT CAT TTT CAT GGG TGA CTC TCT TGA AGT CAC CCA TGA AAA TGA AAC G-3'
psiRNA S11 sense oligo	5'-GAT CCG CTT ACA GGT TGT CTG AAT TTC AAG AGA ATT CAG ACA ACC TGT AAG CTT TTT TGG AAA-3'
psiRNA S11 antisense oligo	5'-AGC TTT TCC AAA AAA GCT TAC AGG TTG TCT GAA TTC TCT TGA AAT TCA GAC AAC CTG TAA GCG-3'
psiRNA S16 sense oligo	5'-GAT CCG ACC AGT CAT GCC TAG ATA TTC AAG AGA TAT CTA GGC ATG ACT GGT CTT TTT TGG AAA-3'
psiRNA S16 antisense oligo	5'-AGC TTT TCC AAA AAA GAC CAG TCA TGC CTA GAT ATC TCT TGA ATA TCT AGG CAT GAC TGG TCG-3'

Recombinant DNA Techniques

In-vitro Ligation of DNA Fragments

Ligation of DNA fragments was conducted as described in section **T4 DNA ligase.** Aside from the usual ligation procedure, if a particular cloning reaction proved to be exceedingly difficult, the use of alkaline phosphatase was employed on the vector generated by restriction enzymes to increase probability of vector-insert ligation. Where appropriate, a

restriction enzyme reaction was conducted post-ligation if vector religation yielded a unique restriction site to increase the likelihood of success.

Transformation of *E. coli*

The method of Mandel and Higa (1970) for transforming *E. coli* cells using $CaCl_2$ as modified by Goodman and MacDonald (1979) was used to introduce recombinant plasmids into bacterial cells.

Preparation of Competent Cells

An overnight inoculum of the appropriate bacterial cell line was grown in LB broth at 37°C. This was diluted 50-fold using fresh broth and grown with vigorous shaking until the OD_{600} was between 0.4-0.5. The culture was transferred into 50mL plastic centrifuge tubes, cooled on ice for 15 minutes, and cells were sedimented by centrifugation using an IEC centrifuge (3000 rpm, 4°C). The cell pellet was resuspended in 20mL of cold transformation buffer containing 75mM $CaCl_2$ and 5mM Tris-Cl, pH 7.5 and incubated on ice for 1 to 16 hours to make competent cells. The cells were collected again by centrifugation and resuspended in 2mL of transformation buffer. At this stage, competent cells were either used directly for transformation or sometimes frozen in 10% glycerol at –70°C and stored in 150µL aliquots in Eppendorf tubes. However, reduction in transformation efficiency by as much as 70% was observed when frozen cells were used, so that when DNA was limiting, freshly prepared competent cells were preferred.

Transformation of Competent Cells

0.2mL of a competent bacterial suspension was used for transformation with ligation reactions not exceeding 40μL volume. When frozen cells were used, the cells were thawed at 37°C and placed on ice immediately. The transformation mixture was added to the Eppendorf tube of competent cells and incubated on ice for 30 minutes to allow the cells to take up DNA. Tubes were gently mixed every 10 minutes to ensure the DNA and the competent cells were well mixed. To facilitate uptake of DNA, the bacterial cells were heat-shocked at 42°C for 45 seconds and placed back on ice for a further 2 minutes. 750μL of LB broth was added and the cells were incubated at 37°C with shaking for 45 minutes to allow for recovery and expression of antibiotic resistance gene(s) before plating on selection media.

Selection

Selection plates contained the required medium with 2% agar (Bioshop) and the appropriate antibiotics. The molten agar was allowed to cool to a temperature between 45°C to 55°C before the addition of the antibiotics. The plates were then poured and allowed to solidify. The agar plates were stored at 4°C and pre-warmed at 37°C just prior to use. When Xgal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) was required, 40μL of 2% solution in dimethylformamide (DMF) was spread onto each plate prior to use.

Suspensions containing transformed bacteria were plated either directly (after pre-incubation at 37°C) or from appropriate dilutions. Dilutions were made so that transformed colonies on selector plates were well separated; that is, the colony count did not exceed about 150 colonies per plate. The plates were incubated at 37°C.

Analysis of Transformants

Individual colonies were picked with a sterile wooden stick and were used to inoculate 2.0mL fresh LB broth in a sterile test-tube. These were incubated at 37°C with vigorous shaking for 4-16 hours. About 1.5mL of the culture was transferred to a fresh Eppendorf tube and the rest was maintained at 4°C for temporary storage. The complete analysis of the transformants was carried out as quickly as possible to ensure that the bacterial culture stored at 4°C remained viable. After analysis and verification, which in many cases was done through restriction enzyme digestion and gel electrophoresis, on some occasions, sequencing, the desired transformant was recloned on agar to ensure the homogeneity of the bacterial stock and the plasmid that was to be used in further studies.

Extraction of Plasmid DNA

The procedure used for extracting plasmid DNA was the alkaline SDS lysis technique of Birnboim and Doly (1978). The protocol, with a number of minor modifications, was used to obtain relatively pure plasmid DNA preparations from small-scale cultures for sequencing as well as molecular cloning.

A 1.5mL culture transferred into a plastic Eppendorf tube was pelleted using a bench-top microcentrifuge at 14,000g for 30 seconds. The supernatant was discarded and the pellet was inverted to allow drying for 2 minutes. The pellet was resuspended by vortexing with 100μL of buffered lysozyme solution (10mM Tris-Cl pH 8.0, 50mM glucose, 50mM EDTA, stored at 4°C as pre-mixed solution with the lysozyme added just prior to use at a concentration of 10mg/mL w/v) and the resuspended pellet was incubated on ice for 15 minutes. Alternatively, 100uL of Resuspension solution (50mM Tris-Cl pH 7.5, 10mM

EDTA, 30mg/mL RNase A) was added in place of the buffered lysozyme solution, and the bacterial suspension was incubated at 37°C for 15 minutes.

200μL of alkaline SDS (1% SDS and 0.2N NaOH) were added and mixed gently by inverting the tube several times. When properly mixed, the reaction mixture would turn relatively clear almost immediately or within 5 minutes during incubation on ice. The mixture was neutralized by adding 150μL of 3M sodium acetate (pH 4.8, pre-mixed and stored at 4°C) and mixed by quickly vortexing which resulted in the precipitation of cellular components and denatured macromolecules within a few seconds. Vortexing the mixture aided in breaking the precipitate into finer particles which were easier to separate from the supernatant during centrifugation. To ensure complete precipitation of cellular protein and nucleic acid, the suspension was left on ice for at least 1 hour, and at times overnight if the DNA preparation was intended for use in sequencing. Prolonged incubation at this stage reproducibly reduced the amount of contaminating RNA in the crude preparation.

The pellicle was separated by centrifugation in a microcentrifuge for 5 minutes and the clear liquid phase was transferred to another tube. To precipitate the DNA, 1mL of 95% EtOH (-20°C) was added and mixed by inverting the tube. This was immediately centrifuged for 5 minutes. The ethanol was removed by aspiration and the nucleic acid pellet at the bottom of the tube was dissolved in 100μL of autoclaved doubly-distilled water. This was precipitated a second time by adding 200μL of cold ethanol and again immediately centrifuged. The pellet was dried for 5-10 minutes at 37°C and dissolved in 100μL of autoclaved TE buffer (pH 8.0) and stored at 4°C for short-term storage to avoid repeated cycles of freeze-thaw. For long term storage, the DNA was stored at -20 °C.

Screening with Restriction Enzymes and Gel Electrophoresis

Restriction Digests

Plasmid DNA extracted from a 1.5mL inoculum was analyzed by restriction enzymes and agarose gel electrophoresis. The method for digesting crude plasmid DNA extract is described in section **Enzymes – Restriction Enzymes**. The digestion (in 25μL) was stopped by either heating to 65°C for 20 minutes for heat labile enzymes or adding 5μL of 6X stopper-gel loading buffer containing 10mM Tris-Cl pH 8.0, 1mM EDTA, 20% sucrose, 0.1% bromophenol blue and 1% SDS (prepared by boiling for 15 minutes and stored at room temperature).

Gel Electrophoresis

Tris-acetate EDTA (TAE) containing 40mM Tris-acetate (pH 7.9), 5mM sodium acetate and 1mM EDTA was the gel electrophoresis buffer of choice employed in this study. A 50X stock was prepared and the appropriate dilutions made prior to use.

Agarose Gels

Appropriate amounts of agarose (Bioshop Biotechnology Grade) were mixed with 1X electrophoresis buffer containing ethidium bromide (0.1μg/mL w/v) and boiled until completely dissolved. (For example, 1.0g of agarose is dissolved in 100mL of 1X TAE buffer to yield a 1% agarose gel). The solution was allowed to cool to 44°C before pouring. The gel was run submerged in 1X TAE buffer in an apparatus so that the buffer recirculated. Generally, the voltage applied across the electrodes ranged from 10V/cm to 20V/cm,

depending on the application. Upon completion, as indicated by the position of the loading dye, the gel was viewed under UV (ultraviolet) illumination and captured using a Kodak Electrophoresis Documentation and Analysis System 120.

Denaturing Agarose Gels

Denaturing agarose gel electrophoresis is a technique associated with either RNA- or single stranded DNA isolation. In this case, the integrity of RNA was verified by visualizing 18S and 28S ribosomal RNA on a denaturing agarose gel. Aside from component differences, samples were generally boiled to denature any secondary structures associated with single stranded regions. To prepare the RNA samples for electrophoresis, 20μL of formamide, 7μL of formaldehyde, 4μL of 10X MOPS buffer (200mM MOPS, pH 7.0, 80mM Sodium Acetate and 10mM EDTA, pH 8.0 in DEPC treated distilled water) and 5μL of RNA loading buffer were added to 15μL of each RNA sample. The sample was then heated to 65°C for 5 minutes and immediately chilled on ice until electrophoresis. A 1.5% agarose gel was prepared by dissolving 1.5g of agarose in 72.5mL DEPC treated distilled water. 10mL of 10X MOPS buffer was also added. The mixture was then heated until the agarose was dissolved and cooled to approximately 45°C. 17.5mL of formaldehyde and 5μL of 20mg/mL ethidium bromide were added to the cooled gel solution and poured into casting tray. Chilled RNA samples were loaded onto the solidified gel and run in 1X MOPS buffer usually at 10V/cm. Post electrophoresis, the RNA denaturing gel was destained by placing the gel in a container of TE buffer made with DEPC treated water. Destaining occurred until the 18S and 28S RNA bands were clearly visible with minimal amount of background. The picture was captured by the Kodak gel documentation system.

Polyacrylamide Gels

SDS-Polyacrylamide gel electrophoresis (PAGE) for protein analysis was prepared at appropriate acrylamide concentration (5%-15% w/v) using a 30:1 ratio of acrylamide to BIS (Sambrook *et al.*, 1989). Appropriate amounts of acrylamide and BIS were dissolved in the buffer of choice and filtered using a 0.45μm Nalgene filter. Immediately before pouring, ammonium persulfate (prepared as a 10% concentrate and stored at 4°C) was added to a final concentration of 0.1% (w/v), and 200μl of N', N', N', N' – tetramethylethylenediamine (TEMED) to initiate polymerization. After pouring, the gel was allowed to polymerize completely for a period of 1 hour and covered with isobutanol. After the polymerization, a stacking gel (usually 2.5%) was prepared on top of the running gel. After the polymerization of the stacking gel, the PAGE gels were used immediately thereafter without pre-running. For samples that are used solely for SDS-PAGE, the protein samples are prepared by boiling in protein loading dye (125mM Tris-HCl pH 6.8, 10% 2-mercaptoethanol, 10% SDS, 10% glycerol and bromophenol blue) for 10 minutes. The samples are cooled in an ice bath before loading onto the SDS gel.

The electrophoretic buffer used is Tris-glycine electrophoresis buffer (25mM Tris, 25mM glycine (pH 8.3) and 0.1%SDS) SDS-PAGE gels are generally run at 15V/cm until the migration front is one quarter of the gel length from the end by visual inspection.

The gel was generally stained with Coomassie brilliant blue solution (Coomassie Brilliant Blue R250 0.25g in 45:45:10 v/v/v methanol: water: glacial acetic acid) for 4 hours. The gel was destained with the wash solution (45:45:10 v/v/v methanol: water: glacial acetic acid) overnight with changes every 3 hours.

Batch Preparation of Plasmid DNA

Plasmid Extraction

Plasmid DNA, when needed in large quantities, was extracted using a scaled-up alkali-SDS procedure (Birnboim and Doly, 1979). 2mL of bacterial stock was added to 500mL of LB broth supplemented with the appropriate antibiotic and incubated overnight at 37°C with vigorous shaking. On occasion, chloramphenicol was added when the optical density (OD_{600}) was between 0.5 and 0.6 resulting in increase in plasmid yield. After incubation overnight, the bacterial culture was transferred into a 250mL plastic tube and centrifuged at 5000 rpm in a Beckman JA-14 rotor (4°C). After decanting the supernatant, the cell pellet was resuspended in 10mL of Resuspension solution using a pipet. After incubation at 37°C for 20 minutes, 10mL of Alkali SDS was added and mixed by gently swirling. The mixture usually turned relatively clear and viscous indicating complete lysis of the cells. After 5 minutes incubation on ice, 10mL of acidic sodium acetate was added and mixed by shaking the tube 1-2 times so the pellicle that formed broke into fine pieces. This was incubated for at least 1 hour on ice. The relative purity of crude DNA extract appeared to be correlated with incubation time. The insoluble pellicle was removed by filtration through sterilized coffee filters with no prior centrifugation of the suspension. The liquid portion was transferred to a fresh tube and 100mL of cold 95% EtOH was added. After mixing thoroughly, the nucleic acids were pelleted by centrifugation at 13800 rpm for 15 minutes in the JA-14 rotor (Beckman J2-MI). The pellet was resuspended in 10mL of sterile TE buffer (50mM Tris-Cl pH 8.0 and 10mM EDTA) and at least 2 volumes of cold EtOH (-20°C) was added for the second precipitation. The tube was once again centrifuged as above and the pellet resuspended in the appropriate amount of TE buffer (approximately 8.5mL).

CsCl-Ethidium Bromide Isopycnic Centrifugation

The crude DNA preparation was further purified by cesium chloride and ethidium bromide centrifugation. To the resuspended DNA pellet, approximately 10g of cesium chloride (Bioshop) were added and mixed thoroughly until completely dissolved. The density was adjusted by adding more cesium chloride until it reached 1.58-1.60g/mL (checked by weighing 1mL of solution). The solution was transferred into a Beckman ultracentrifuge tube and 0.5mL of 5% (w/v) ethidium bromide was layered on top. The tube was sealed by the appropriate stopper and care was employed to avoid any air bubbles. The resulting tubes were centrifuged in a Beckman L-series Ultracentrifuge for 22 hours at 60,000 rpm at 20°C in an NVT65 rotor.

The DNA band that migrated at a higher density was extracted by puncturing the side of the tube with an 18G needle and collected with a disposable syringe. The band, when intense, could be seen in ordinary light. The ethidium bromide was extracted repeatedly with 2 or more volumes of CsCl-saturated TE-isoamyl alcohol until the no traces of pink were left in the aqueous phase (bottom layer). Finally, the DNA solution was extensively dialyzed against 0.1X SSC buffer (prepared as a 20X stock containing 3M NaCl and 0.3M sodium citrate). The buffer was changed after 1 hour, and then again every 24 hours for 2 days. After dialysis, the DNA preparation was stored at –20°C for extended storage or at 4°C if to be used frequently.

DNA Sequence Analysis

The sequencing of the appropriate clones was done using Visible Genetics Inc.'s Cy™5.5 Dye Primer kit along with the Opengene™ automated DNA sequencing system. The sequencing was performed as per manufacturer's protocol, using primers 291F and 552R, with the following cycling conditions:

Table 5: Cycling conditions for DNA sequencing reaction

Cycle(s)	Temperature	Time
1	95°C	3 minutes
30	95°C	1 minute
	54°C	1 minute
	72°C	1 minute
1	72°C	7 minutes

Purification of DNA from Agarose Gels

Restriction fragments greater than 300bp were fractionated by agarose gel electrophoresis and purified using a) commercially supplied GeneClean® kit from Bio101 or b) in-house gel purification kit GelSelect from Norgen. Detailed procedure followed as per manufacturer supplied protocol. Desired fragments were manually excised from the agarose gel and the volume was determined by weight. Three to five volumes of 6M sodium iodide were added to the gel slice and heated to 55°C for 5 minutes to melt the gel slice. Visual confirmation is performed to ensure that gel slice has completely melted. The melted gel solution was loaded onto the binding columns and centrifuged at 14,000 rpm for 1 minute, and flow-thru was discarded. 400μL of the wash solution were added to the column and centrifuged at 14,000 rpm for 1 minute. Flow-thru was once again discarded. Finally, 50μL to 100μL of elution buffer were added to elute the DNA from the binding matrix.

Determination of DNA concentration

For many applications, the precise concentration of DNA stocks was not absolutely required. For example, standard cloning techniques allow a wide range of near-optimal

conditions that quite often guarantee the success of obtaining the proper clones without the need to know the precise DNA concentrations of reactant fragments. However, in cases such as transfection assays, knowledge of the concentration of plasmid DNA used was necessary for quantitative calculations. The following combination of various methods to determine DNA concentration was applied for various uses.

Optical Density determination of DNA concentration

First, the concentrations of DNA stocks purified by cesium chloride and ethidium bromide isopycnic centrifugation technique were approximated by obtaining the optical density of a diluted solution at 260nm. In the second step, the determined OD_{260} was used to prepare a diluted DNA stock at 100ng/μL. In cases where purity had to be assessed, the OD_{280} was also determined and its relative purity determined by the ratio of OD_{260} to OD_{280} as according to Sambrook *et al.* (1989).

Diphenylamine Method

In cases where purification was performed using Norgen's DNA binding resin, the concentration of DNA could not be obtained by optical density due to contaminating micro-particles. As such, the absolute concentration in these preparations was then determined by the diphenylamine method using a standard DNA solution of salmon sperm DNA whose concentration was determined from its optical density at 260nm wavelength.

The modified diphenylamine assay was adapted from Giles and Myers (1965). Since this was a colorimetric reaction assay, the concentration was expressed as an interpolated value from a standard curve established by a simultaneous assay with known amounts of

salmon sperm DNA. Thus, the DNA sample was diluted in doubly distilled water to a final volume of 250µL in a glass disposable test tube. Using a Pipetman®, 250µL of 20% perchloric acid was added and mixed by shaking the tube slightly. Immediately after, 500µL of 4% diphenylamine (w/v in glacial acetic acid) was added and mixed. To this 50µL of 0.15% acetaldehyde was added. The samples were incubated overnight at room temperature to allow a complete reaction to occur. The absorbance of the samples was determined at wavelength 595nm using a Beckman® DU7 Spectrophotometer. Although a single wavelength reading at 595nm was normally sufficient, the results were invariably improved if a second reading at 700nm was subtracted from the first value.

The standard curve which was used to determine the unknown values was established using Microsoft Excel. Thus, the paired data (amount of DNA vs. OD_{595}-OD_{700}) were entered and the regression line was calculated. The reliability of the assay was assessed by the correlation coefficient (R^2), in which case 0.95 was considered acceptable; otherwise, the assay was repeated.

Mammalian Cell System

Three mammalian cell lines were used in transient expression assays: HeLa, HEK 293 and MDBK cells. HeLa cells were grown in α-minimal essential medium (α-MEM) from Gibco BRL® supplemented with 10% donor horse serum (obtained commercially through Cansera) and 1% Glutamax™, which was a commercially available dipeptide of l-ala-l-glu. For prophylactic purposes, 100X antibiotic/antimycotic solution was obtained from Gibco BRL®, which, at working concentration, contained penicillin G sodium (100 U/mL), streptomycin sulphate (100 µg/mL), and amphotericin B (2.5 µg/mL). HEK 293 cells were obtained from the American Type Culture Collection and were grown in α-MEM

supplemented with 10% fetal bovine serum and the above supplements. MDBK cells were also grown in α-MEM with similar supplements. The only difference was that 10% bovine serum was used instead of fetal bovine serum.

Growth in Tissue Culture Dishes

All cells were propagated as monolayers in Sarstedt 150mm culture dishes and maintained in 37°C incubators with 96% humidity and 5% CO_2. HeLa and MDBK cells were passaged as follows. Cells were checked until they reached approximately 70% confluence. The spent medium was removed by vacuum aspiration and the cells were washed with 5mL of versene (per Litre: 1.0g glucose, 0.4g KCl, 8.0g NaCl, 0.58g $NaHCO_3$ and 0.2g EDTA) once. 0.025% Trypsin (Gibco) in versene was added at 3mL/dish and incubated at 37°C for 2 minutes. After the cells in culture had partially detached (as indicated by rounding of the cells) from the bottom of the dish, the cells were completely dislodged by slightly tapping the side of the dish. Fresh medium was added, as proteins in serum inhibit trypsin activity, and cells were evenly distributed in a 1:2 fashion to fresh culture dishes. To avoid possible damage to delicate HEK 293 cells, the following modification was made. The HEK 293 cells were washed and incubated in 1X saline-citrate (150mM NaCl, 15mM sodium citrate, pH 7.0) without the addition of trypsin.

Maintenance of Cells Used in Transfection Assays

The maintenance of cells used in transfection assays required more care to minimize variability that is often associated with this type of assay. First, cells were never allowed to reach full confluence, which greatly affected the rate at which the cells would take up DNA.

Secondly, trypsinized cells were counted in a haematocytometer to determine the concentration of cells to be seeded to reach approximately 50% confluence in the following day. However, the seeding concentration varied between cell lines and was determined empirically since the doubling rate of each cell line differs greatly. Third, cells were typically trypsinized for a shorter duration so as to avoid the possibility of cell damage during incubation. Finally, α-MEM supplemented with 10% fetal bovine serum was used for all cells the passage before the transfection assays.

Transfection Assays

Preparation of Carrier DNA

High molecular weight DNA (genomic) used in transfection studies was extracted from cell monolayers. Cells close to confluence were washed with sterile distilled water or PBS^-, and to each 150cm dish, 5mL of SDS-pronase solution (0.5mg/mL pronase in a buffer containing 10mM Tris-Cl (pH 7.5), 5mM EDTA and 0.1% SDS) was added. The solution was spread evenly by tilting the dish several times, and then the dish was placed in a cell culture incubator for 15 minutes. The mixture from the dishes was scraped using a sterile silicon-rubber policeman and transferred into a 50mL plastic centrifuge tube for further incubation (3-16 hours) at 37°C. Alternatively, incubation was done at 52°C for a shorter duration (1-3 hours). The mixture was extracted twice with one volume of buffered-saturated redistilled phenol (with 0.1% 8-hydroxyquinoline) and once with one volume of water-saturated chloroform to remove residual phenol. The aqueous phase was recovered and placed in a beaker to which sodium acetate (pH 8.0) was added to a final concentration of 0.2M. To precipitate the DNA, greater than 2 volumes of ethanol (-20°C) was poured slowly

into the isolated aqueous phase. The solution was mixed gently and a glass pipette was used to assist in the spooling of the DNA. The spooled DNA was then dried briefly and dissolved in TE buffer (pH 8.0). Short term storage of carrier DNA occurred at 4°C, while long term storage was at -70°C.

Prior to transfection, the genomic carrier DNA was sheared by passing it through a fine needle (20G) and syringe.

Transfection Using the Calcium Phosphate Technique

Transfection assays using the calcium technique (Graham and van der Eb, 1973) were carried out using HeLa, 293 and MDBK cells. The transfection cocktail was prepared as follows. Into one 15mL centrifuge tube 2mL of 2X HEPES-buffered saline (2X HEBS, prepared by dissolving 8.0g NaCl, 0.37g KCl, 0.1g Na_2HPO_4, 5.0g HEPES and 1.0g glucose in 500mL distilled water; adjusted to pH 7.1; autoclaved, stored at 4°C), was dispensed at room temperature. In a separate tube, the plasmid and carrier DNA were mixed together and the final volume was adjusted to 1.5mL with TE (pH 8.0) buffer. The amount of carrier DNA used was empirically determined according to the method of Graham and van der Eb (1973). To this tube, 0.5mL of 1M $CaCl_2$ was added, mixing the solution well. After a brief low-speed centrifugation to collect the solution at the bottom of the tube, the solution was added dropwise to the other tube containing 2X HEBS with constant swirling. A fine precipitate was formed and the size of precipitate correlated to the success of the assay, with finer precipitate resulting in better transfection efficiency; the cocktail was allowed to stand at room temperature for 10 minutes prior to addition to the cells. Three to four hours after the addition of DNA, the medium was replaced with fresh α-MEM supplemented with 10%

fetal bovine serum. Approximately 24-72 hours after the initial addition of DNA, the cells were analyzed by a variety of assays (see below).

Preparation of soluble protein extract

Freeze-and-thaw technique

Cells were washed once with 5mL of ice-cold PBS^- and aspirated. To collect all the cells from the culture dish, 1mL of PBS^- was added and the culture dish surface was scraped with a silicon rubber policeman. The resultant cell suspension was transferred into a 1.5mL Eppendorf tube. Cells were collected to the bottom by a 10 second centrifugation at 12,000g and the cell pellets were either immediately used or kept at –70°C until needed.

To prepare soluble protein extracts, the cell pellet from one dish was resuspended in 200µL of freeze/thaw buffer (FT; 10mM Tris-Cl pH 7.4, 10mM EDTA, 25mM sucrose) by vortexing for several seconds. Cells were broken by four cycles of freezing in liquid nitrogen (30 seconds) and thawing in a 37°C water bath (5 minutes). Cellular debris was separated from the cytoplasmic extract by centrifugation for 5 minutes at 4°C using a bench-top microcentrifuge. The clear supernatant was transferred into a fresh tube and was used immediately or kept at –70°C until needed.

Single Detergent Lysis

The method of extracting soluble proteins from cells was performed essentially as described by Sambrook *et al.*, 1989. Cultured mammalian cells were grown to near confluence. The medium was aspirated, and cells were washed three times with ice-cold 1X

PBS$^-$. To the cells, 2ml of the single-detergent buffer (50mM Tris-Cl pH 8.0, 150mM NaCl, 0.02% sodium azide, 100μg/mL PMSF, 1μg/mL aprotinin and 1% Nonidet P-40) was added and incubated on ice for 20 minutes. After the incubation, cells were scraped with a rubber policeman. Using a pipette, the cell debris and lysis buffer were transferred to a chilled Eppendorf tube. The lysate was centrifuged at 12,000g for 2 minutes at 4°C. The supernatant was transferred to a fresh Eppendorf and stored on ice before proceeding to the next step. Long-term storage of the soluble protein extract occurred at -70°C.

Quantitation of Protein Concentration

The BioRad protein assay, which was based on the Bradford assay (Bradford, 1976), was used to determine the concentration of proteins in the various soluble protein extracts. In this assay, the standard curve was established using known amounts of bovine serum albumin (BSA) that were commercially obtained from NEB (10mg/mL BSA as supplied with Restriction Enzymes) to construct a standard curve. The concentrations of protein samples were determined based the standard curve of known protein concentration. While this method sufficed in the determination of protein concentration isolated via the freeze-thaw method, the well-documented interference of detergents causing overestimation of protein concentration prompted the use of an alternative means of quantification.

BioRad Assay

The BioRad assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 due shifts from 465nm to 595nm when binding to protein occurs (BioRad Assay manual).

The dye reagent was prepared by diluting the Dye Reagent Concentrate 5-fold with distilled water. The solution was filtered to remove particulates, and 200μL of the Dye Reagent is added to 800μL of unknown protein samples and vortex briefly. The solution was incubated at room temperature for 5 minutes and absorbance reading was taken at 595nm. As stated above, the concentration of the protein is determined by comparing to the standard curve constructed using known amounts of a protein standard (i.e. BSA).

Bicinchoninic Acid (BCA) assay

The principle of the bicinchoninic acid (BCA) assay is similar to the Lowry procedure, in that both rely on the formation of a Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{+}. The amount of reduction is proportional to the protein present. BCA forms a purple-blue complex with Cu^{+} in alkaline environments, thus providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins at absorbance maximum of 562 nm (Sigma BCA protein assay manual). An advantage of the BCA method of protein determination is its tolerance to a host of common laboratory detergents such as Triton X-100, Tween 20 and Nonidet P-40.

To prepare the BCA protein dye solution, one volume of copper sulfate solution was diluted with 50 volumes of BCA solution. 2mL of the BCA protein dye solution were added 100μL of protein samples and incubated at room temperature for 30 minutes. The resulting absorbance was taken at 562nm. As stated above, the concentration of the protein was determined by comparing to the standard curve constructed using known amounts of a protein standard (i.e. BSA).

Enzymatic Assays

A popular and inexpensive method for assaying β-galactosidase activity in cell extracts is a colorimetric method based on the hydrolysis of o-nitrophenyl-β-D-galactopyranoside (colorless) into a yellow product o-nitrophenolate (Miller, 1972).

To assay for β-galactosidase activity, 50μL of the total extract was added to a reaction mixture containing 150μL of Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$ and 50mM β-mercaptoethanol, adjusted to pH 7.0) and 40μL of 4mg/mL o-nitrophenyl-β-galactopyranoside (ONPG from Sigma, in 60mM Na_2HPO_4, 40mM NaH_2PO_4 in distilled water; stored in small aliquots at –20°C), and incubated at 37°C. The reaction was stopped by adding 100μL of 1M Na_2CO_3 normally after 30-60 minutes incubation. The extent of the reaction was quantified by determining the spectroscopic absorbance at 420nm and the specific activity was calculated using the following formula:

$$\text{Specific Activity (Units/mg)} = \frac{A_{420}/0.0045}{\text{Incubation time (mins)} * \text{Amount of protein (mg)}}$$

Plasmid digestion assay

To assess the amount of DNase activity in the various soluble protein extract from various cell lines, a plasmid digest assay was devised. Plasmid DNA, pCMV-β, was incubated with various soluble protein extract obtained in section Single Detergent Lysis. The plasmid vector pCMV-β was first cut with the restriction enzyme *HindIII* to allow for a more accurate densitometry reading. The concentration of each of the soluble protein extract was determined by either the Bradford assay or the BCA assay, and subsequently normalized

to the same concentration by preparing dilutions with the triple detergent lysis buffer. After inactivating *HindIII* as per manufacturer's protocol, approximately 0.5μg of *HindIII*-linearized pCMV-β was added to the normalized soluble protein extracts and incubated at 37°C for 30 minutes. The reaction was terminated by phenol-chloroform extraction, and the aqueous layer was assessed by agarose gel electrophoresis. Staining of the agarose gel with ethidium bromide occurred after gel electrophoresis was completed. Densitometry readings were obtained by either BioRad densitometry software or Alphaimager AlphaEaseFC.

Inhibitors of plasmid digestion assay

Various inhibitors, as described in chapter III, were added to examine the effects of inhibitors to differentiate the various DNases based on biochemical characteristics. Essentially, the assays were the same as above except for the addition of the inhibitors. However, additional controls were performed to ensure that the effects of the plasmid digestion assay were solely caused by DNases and not instability of the plasmid in the presence of the inhibitors. The same amount of plasmid was left to incubate under identical conditions except the soluble protein extracts were not included. Similar densitometry analysis was performed for the inhibitor incubation control. Where the inhibitors were found to affect the stability of the plasmid, the disappearance of the plasmid was calculated to give a mathematical normalization curve to use to subtract the effects of the degradation by DNase activity from the effects of the incubation with the inhibitor alone.

Rapid Cloning of cDNA ends (RACE)

RNA isolation

Total RNA was extracted from MDBK cells using Norgen RNA binding kit (beta). Culture dishes of MDBK cells grown to 75% confluence were washed three times with 5mL of ice cold PBS. After the PBS was aspirated, cells were lysed by adding 2mL of Norgen RNA Lysis buffer (4M guanidine HCl, 0.025M sodium citrate, 0.25% NP-40) and were incubated, on ice, for 10 minutes. The Norgen RNA binding columns were activated by adding 400μL of Activation buffer (4M guanidine HCl) and collected via centrifugation (14,000g for one minute). The total lysates were added to the column and collected. The bound total RNA was washed twice with the Wash solution (70% EtOH, 1mM EDTA and 10mM Tris-Cl). Total RNA was eluted with 100μL of the Elution buffer (5mM Tris-Cl, 1mM EDTA, pH 9.0). The integrity of the extracted RNA was verified by the denaturing agarose gel electrophoresis. When the 18S and 28S RNAs were clearly visible without smearing, the RNA was deemed to be fit to carry onto the cDNA synthesis (Refer to the section under **Denaturing Agarose Gels**).

Cloning of DNase γ using 5' RACE

The RACE procedure was carried out as described by the Ambion RLM-RACE kit. The procedure contained numerous modifications to the PCR-based amplification as described by Frohman *et al.*, 1988. To enhance the likelihood of success of 5' RACE, a few enzymatic reactions were performed. Total RNA was treated with Calf Intestine Alkaline phosphatase (CIP) to remove free 5'-phosphates from molecules such as ribosomal RNA, fragmented mRNA, tRNA and contaminating genomic DNA. The pyrophosphate cap that

typically accompanies that of mRNA is unaffected by CIP. The RNA was then treated with Tobacco Acid Pyrophosphatase (TAP) to remove the cap structure from full-length mRNA, leaving a 5'-monophosphate. Since other contaminating species have been altered by CIP, the only 5'-monophosphate that is needed for ligation with a 45 bp RNA Adapter oligonucleotide should have stemmed from only non-degraded, full length mRNA. After RNA ligation using T4 RNA ligase, first strand cDNA was obtained by reverse transcription reaction using random hexamers. The cDNA was then subjected to nested PCR using a 5' RACE primer complementary to the RNA adapter and a gene specific primer in each PCR amplification.

Cloning of DNase γ using 3' RACE

First strand cDNA was synthesized from total RNA, using a 3' RACE adapter. The cDNA was then subjected to PCR using one of the 3' RACE primers which were complementary to the anchored adapter, and a gene specific primer.

Reverse Transcription and cDNA synthesis

Reverse transcription of RNA was performed using Superscript II reverse transcription kit from Invitrogen. Up to 5μg of RNA (in 5uL) were mixed with 1μL of random decamers (or 3' RACE adapter for 3'RACE applications) and heated to 70°C for 10 minutes. The RNA-primer solution was then immediately put on ice. To the solution, the following were assembled: 2μL of 5X first-strand buffer (250mM Tris-HCl, pH 8.3 at RT, 375mM KCl, 15mM $MgCl_2$), 1μL of 0.1M DTT and 1μL of dNTP mix (10mM each of dATP, dCTP, dGTP, dTTP). The mixture was vortexed briefly and incubated at 42°C for 2 minutes. 0.5μL of Superscript II was added and incubated at 42°C for 50 minutes.

Polymerase Chain Reaction (PCR) amplification

PCR amplification was performed using a MJ MiniCycler. Generally, the conditions varied slightly depending on the primer pair melting temperature and expected size of PCR product. For detailed examination of the cycling conditions, please refer to http://info.med.yale.edu/genetics/ward/tavi/Guide.html. To increase likelihood of success, a technique known as Hot Start PCR was performed. In a PCR tube, the following reagents were assembled: 1-2μL of DNA sample, 5μL* of 10X PCR buffer (750mM Tris-HCl (pH 8.8 at 25°), 200mM $(NH_4)_2SO_4$, 0.1% Tween 20), 5μL* of 25mM $MgCl_2$, 1μL of 10mM dNTP mix, 2μL* of each PCR primer (15mM stock) and MilliQ water to 45μL total volume. 5μL of sterile glycerol was pippetted on top of the reagents and placed in the thermocycler, where the thermocycling program began (see below). In another Eppendorf tube, 1μL of *Taq* polymerase was added to 4μL of MilliQ water and placed on ice. After the first denaturation step (3 minutes), the thermocycler was paused, and the *Taq* polymerase solution was added quickly to each reaction. The typical thermocycling conditions were shown in Table 6:

* Amount to be optimized for each PCR reaction

Table 6: Cycling conditions for a typical PCR reaction

Cycle(s)	Temperature	Time
1	95°C	3 minutes
30	95°C	1 minute
	54°C	1 minute
	72°C	1 minute
1	72°C	7 minutes

Chapter III

Factors affecting transfection efficiency in mammalian cells

The cell cycle dependence of transfection efficiency

Evidence in the literature had suggested that transfection efficiency in mammalian cells varies greatly depending on the stage of cell cycle (Strain *et al.*, 1985, Yorifuji *et al.*, 1989, Goldstein *et al.*, 1989). Most transfection methods, including some viral vectors, require cells to be dividing to ensure success, which implies the need for cell cycle progression for transfection to occur (Miller *et al.*, 1990). Evidence suggested that transfection efficiency was facilitated during mitosis due to the dissolution of the nuclear membrane, which allowed transfected plasmid access to the nucleus without having to traverse the nuclear pore (Wilke *et al.*, 1996 and Mortimer *et al.*, 1999). Nuclear import of plasmid DNA is an energy dependent process whose efficiency decreases with increasing plasmid size (Hagstrom *et al.*, 1997). It is reported that plasmids greater than 1kb cannot gain access to the nucleus through the nuclear pore complex without the presence of nuclear localization signal, which presents a major barrier as most plasmids require components that are greater than 1kb (i.e. Amp^R gene is approximately 1kb with promoter elements). However, there is also evidence suggesting that the dissolution of nuclear membrane is not necessary for successful transfection to occur, and that nuclear pores allow for the passage of

plasmid molecules of 4.3kb efficiently (Dean, 1997). The issue is one that remains to be resolved.

Initially, examining the correlation between transfection efficiency and cell cycle allowed for the elucidation of gene products that are differentially expressed during the various cell cycles. By correlating genes expressed during the cell cycle that demonstrates maximum transfection efficiency with genes expressed during the cell cycle that demonstrates minimum transfection efficiency, elucidation of genes associated with transfection is possible by subtractive hybridization. However, before subtractive hybridization can be performed, there must be a demonstration of cell cycle dependence of transfection efficiency. Literature report conflicting results in terms of the specific phase of the cell cycle at which maximal transfection efficiency occurs (S-phase: Grosjean *et al.*, 2002 and Giulotto and Israel, 1984 and G2/M phase: Goldstein *et al.*, 1989). Thus, the cell cycle effects were examinded by synchronizing HEK-293 cells and performing transfection at various time points after the synchronization agent is removed. Cell synchronization is achieved by a variety of chemical means, which would lead to the cell cycle arrest of the entire population of cells. Upon the removal of the arresting agent, cell cycle presumably continues as a population and thus the term "cell synchronization", signifying the homogeneity of the cell cycle progression as a population. Subsequently, transfection assays would be performed, thus, establishing the cell cycle that exhibits maximal transfection efficiency. Using a widely accepted method of cell synchronization, HEK-293 cells were treated with the double thymidine block protocol. Thymidine causes G1/S arrest and cell population would enter S phase synchronously after the block is removed (Harper, 2004). However, double thymidine block cannot achieve a high state of cell-cycle synchrony in the cell population (Cao *et al.*, 1991). This effect, coupled with the usual noise associated with the β-galactosidase based transfection assay, complicated the analysis of normal transfection

data. To remedy this problem, a newer protocol was adopted in which greater than 90% of cell synchrony was reported by a double thymidine block, followed by a mitotic shake off. Cells that are obtained by mitotic shake off would be treated further with the DNA replication inhibitor, hydroxyurea (Knehr *et al.*, 1995). Aside from the laborious nature of this synchronization technique, transfection studies revealed that the additional hydroxyurea, which was intended to improve cell synchronization, completely inhibited transfection, to a point where no β-galactosidase activity was detected within a 6-well plate.

With evidence suggesting the numerous pleotrophic effects of cell synchronization chemicals (Cohen and Studzinski, 1967 and Cooper, 2004), coupled with the difficulty of reconciling the data noise based on the traditional double thymidine approach, the approach of utilizing cell synchronization agents to correlate transfection efficiency with a specific phase of the cell cycle became unattractive. Without establishing a specific phase of the cell cycle exhibiting highest transfection efficiency, elucidation of transfection associated gene became impossible. Thus the approach had to be changed to study these transfection associated genes.

The cell line dependence of transfection efficiency

Lin *et al.,* (1994) demonstrated that cell lines differ in their propensity to be transfected. An approach was devised to elucidate cell line specific genes that are associated with transfection.

With an increase in understanding and development of newer transfection methodologies, transfection efficiency has been significantly enhanced in certain cell lines. However, when compared to viral vectors, non-viral means of gene transfer remain generally low in transformation efficiency. Generally, for the molecular biologist, cell lines are chosen

based on their transfection efficiency and their relevance to the topic of investigation. Then, a vector of gene transfer is chosen depending on the utilization, safety and feasibility. For example, HEK-293 cells are a popular cell line for transfection studies because of their innate propensity to be transfected, even with the traditional calcium phosphate technique. In fact, transfection efficiency has been reported to be as high as 90% of total cell population when transfected with Lipofectamine 2000 (Technical resources from www.invitrogen.com).

However, cell lines that are generally refractory to transfection, such as fibroblasts, yield consistently low transfection efficiency regardless of transfection method (Segura *et al.*, 2001). Due to the technical difficulties encountered examining the cell cycle dependent factors affecting transfection efficiency, it was reasoned that studying the factors that attribute high transfection efficiency in one cell line and low transfection efficiency in another could also reveal insights into general factors that affect transfection efficiency, and perhaps isolate genes that affect transfection. Using commonly available cell lines with established transfection efficiency (with HEK-293 cells transfecting the most efficiently, and MDBK cells transfecting the least efficiently), the differences between cell lines were examined in order to generate a model of how cell line dependent factors affect transfection efficiency. Manipulation of the cell line dependent factors could serve to enhance transfection efficiency of cell lines that are generally refractory to transfection. Thus, an investigation of cell line specific factors that affect transfection efficiency was undertaken.

DNase activity of various cell lines

It was reasoned that transfection efficiency could differ between cell lines in 3 general areas: (1) plasmid DNA degradation, (2) plasmid DNA uptake and (3) expression of transgene. In terms of differential plasmid DNA uptake, the plasma membrane presented a

unique challenge to study plasmid DNA uptake. Furthermore, advancement in DNA delivery methods has overcome the plasma membrane as the determining factor for successful transfection. Examining differential expression of transgene would be exceedingly difficult as it would involve direct introduction of plasmid DNA into the nuclei of cells and evaluate differences in transfection efficiency of various cell lines. Of the three areas, differential plasmid DNA degradation was the easiest to examine. The central assumption that passive diffusion en route to the nucleus after internalization is adequate for expression of transgene is not straightforward. There are numerous choke points that allow DNases access to transfected plasmids. Thus, an approach was devised to examine whether there is a correlation between transfection efficiency and plasmid degradation.

While there is a lot of information about nucleases present in the cell, their exact physiological roles remain somewhat obscure. Other than their use as molecular biology tools, few have examined why cells need such a diverse array of nucleases. Until recently, with the discovery of the role that caspase-activated DNase (CAD) plays in apoptosis (Enari *et al.*, 1997), DNases have been mainly associated with functions in recombination, maintenance of genetic stability and transformation (Baranovskii *et al.*, 2004).

The presence of DNases became a possible explanation to account for differences in transfection levels in a cell-specific manner. Potentially, cell lines that are refractory to transfection may innately express a high amount of DNases, and vice-versa. After all, the conceptual linkage between the stability of plasmid DNA within the cell after endocytosis and transfection efficiency was one of simple logical progression. If nuclease activity was extraordinarily high in a particular cell line, degradation of transfected plasmid would not allow for intact plasmids to find their way into the nucleus. Once internalized, one can envision that the majority of intact plasmids do not survive under the onslaught of the slew of

DNases. As a result, the degradation of plasmid DNA in transition to the nucleus may present a major obstacle in transfection.

To test the validity of the above hypothesis, a traditional technique of obtaining a soluble protein extract, using a single detergent lysis method, was used to assess DNase activity (Sambrook, Fritsch and Maniatus, 1989). Using the reporter plasmid pCMV-β, the disappearance of plasmid DNA due to DNase degradation, upon the incubation in different soluble protein extracts, was measured. Commonly available cell lines were chosen based on their established levels of transfection efficiency, with the HEK-293 cell line generally exhibiting the highest levels of transfectability and MDBK being the most refractory to transfection. The HeLa cell line served to show an intermediate range of transfection efficiency. Cell lines were grown to approximately 50% confluence, which would correspond to the general population density during transfection, and soluble protein extracts were normalized in terms of amount of protein present. It was reasoned that a cell line exhibiting a high level of DNases would so reflect not only on a per cell number basis but also on a per protein concentration basis. Due to the presence of detergents, the BioRad protein assay was replaced by the BCA assay for protein quantification due to observable detergent interference (see Appendix B). The BCA assay is reported to tolerate up to 1.0% of Nonidet-P40 in the protein sample without causing interference with quantitation accuracy (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Proteomics_and_Protein_Expr_/Protein_Analysis/Protein_Quantitation/Bicinchoninic_Acid_Kit.html).

Generally, plasmid integrity is rigorously tested before transfection studies are conducted. Thus, to examine differential plasmid DNA degradation by soluble protein extracts as a means of inferring information about behavior of transfected plasmids, incubation of supercoiled plasmids would be an appropriate choice since it represented more closely the state of transfected plasmids (over linearized plasmids, per se). However, using

the densitometry software, it was discovered that the quantification of intact plasmids and summing their integral areas (two bands representing two conformations of plasmid DNA: nicked and supercoiled) was less accurate than quantification of a singular band stemming from linearized plasmids, possibly due to differential binding of ethidium bromide by conformational effects. Thus, in order to increase our accuracy of plasmid quantification using densitometry, the plasmid, pCMV-β, was linearized with the restriction enzyme *HindIII*. The enzyme was heat inactivated for 20 minutes at 65°C instead of phenol/chloroform extraction as it was feared that phenol/chloroform contamination may inhibit nuclease activities of the soluble protein extracts.

Figure 6: The effects of linearized pCMV-β incubated with the soluble protein extracts obtained from HEK-293, HeLa and MDBK cells (representative gel electrophoresis photo). Temperature of incubation was at 37°C. (**C** – no soluble protein extract control; **1** – time point at 0 minutes; **2** – time point at 30 minutes; **3** – time point at 60 minutes; **4** – time point at 90 minutes; Marker lane – Norgen Biotek FullRanger)

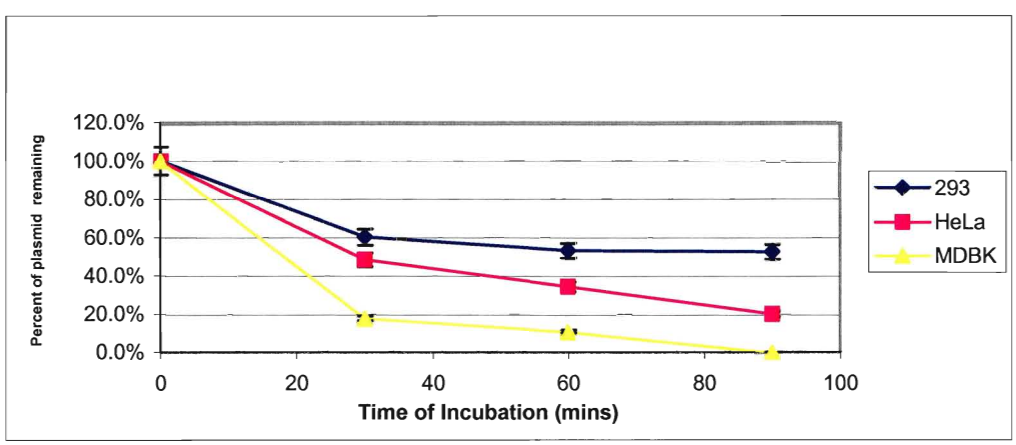


Figure 7: The effects of linearized pCMV-β incubated with the soluble protein extracts obtained from HEK-293, HeLa and MDBK cells using AlphaEaseFC densitometry software. The percent of plasmid remaining is obtained by the expressing the densitometry readings at the various time points as percentages of the control (time point at 0 minute). These are resulting means of three trials conducted simultaneously.

From literature, HEK 293 cells generally exhibit transfection efficiency of 35% (Jordan *et al.*, 1996) with HeLa generally exhibiting around 17% (Chen and Okayama, 1987) and MDBK known for its low transfection efficiency. Using this plasmid digestion assay, we observed that the literature values of transfection efficiency of various cell lines correlated negatively with the cell-line specific DNase activity, which agreed with our hypothesis. While it is uncertain whether the presence of DNases alone can account for all of the difference in their respective ability to undergo transfection, it is reasonable that, *ceteris paribus,* a cell line exhibiting a high amount of DNases would result in the lowering of exogenous plasmid half-life, resulting in lower transfection efficiency.

Following the DNase digestion assay, the intensities of the remaining bands, stained by ethidium bromide, were captured by the BioRad GelDoc system, and the resultant picture analyzed using the densitometry software AlphaEaseFC. Visual inspection of Figure 6 revealed that plasmid stability is lower in the presence of MDBK soluble protein extract. From Figure 7, there was only 20% of the initial amount of plasmid left intact after 30 minutes of incubation with the soluble protein extract from the MDBK cell line. This was sharply contrasted by the results obtained from HEK-293 cells, which showed approximately 60% of the initial plasmid intact, with HeLa achieving an intermediate value of 50%. After 90 minutes of incubation, virtually none of the plasmid remained for MDBK. Thus, it was concluded that the level of DNase activity of the MDBK cell line far exceeded that of the HEK-293 cell line, with HeLa demonstrating an intermediate level of DNase activity. As a preliminary step, these results were consistent with the notion that DNases are involved in reducing transfection efficiency in a cell-line specific manner.

Isolation of the DNase target for further evaluation

Because of the presence of numerous DNases within a cell, the observation that the MDBK cell line contained the highest amount of DNase activity most likely stemmed from the combined effects of various individual DNases (i.e. DNase I, DNase IL2, DNase γ, DNase X, DNase II and DLAD, to name a few). With the eventual knockdown studies in mind, isolation of fewer potential target DNases for further evaluation was to be performed by segregating the possible members based on a variety of characteristics. These characteristics include various chemical inhibitors and biochemical characteristics.

Evaluation of the pH profile of MDBK soluble protein extract

Based on the fact that the MDBK cell line, whose transfection efficiency is the lowest of the group, possesses the highest amount of DNase activity in its soluble protein extract, gene knockdown of a the predominant DNase may enhance transfection efficiency. If that proved to be true, it would provide evidence that DNase activity and transfection efficiency share a causal relationship. However, with a wide range of DNases available in the cells and limited biochemical and sequence information available for some DNases, cloning and gene knockdown of all possible DNases were not possible. Differentiating between the DNases in the soluble protein extracts became a key to determine which DNase is the likely culprit.

From the literature, members of both DNase I- and DNase II family of DNases can be segregated based on certain biochemical characteristics (Shiokawa and Tanuma, 2001 and Evans and Aguilera, 2003). For example, members of the DNase I family of DNases have neutral pH optima while DNase II family members share an acidic pH optimum. Based on the same soluble protein extracts obtained in the previous section, their pH profiles were

examined using a modified version of the plasmid digestion assay, in a cell-line specific manner. During the isolation of soluble protein extracts, the single detergent lysis solution was buffered at pH 8.0. Additional buffers were added to yield a variety of pH values required from pH 4.0 to pH 9.0 during the plasmid digestion reaction. Essentially, pH values outside of the ranges examined were physiologically rare and were thus omitted. Furthermore, plasmid DNA stability is also impacted at extremely high or extremely low pH values, which may skew the results.

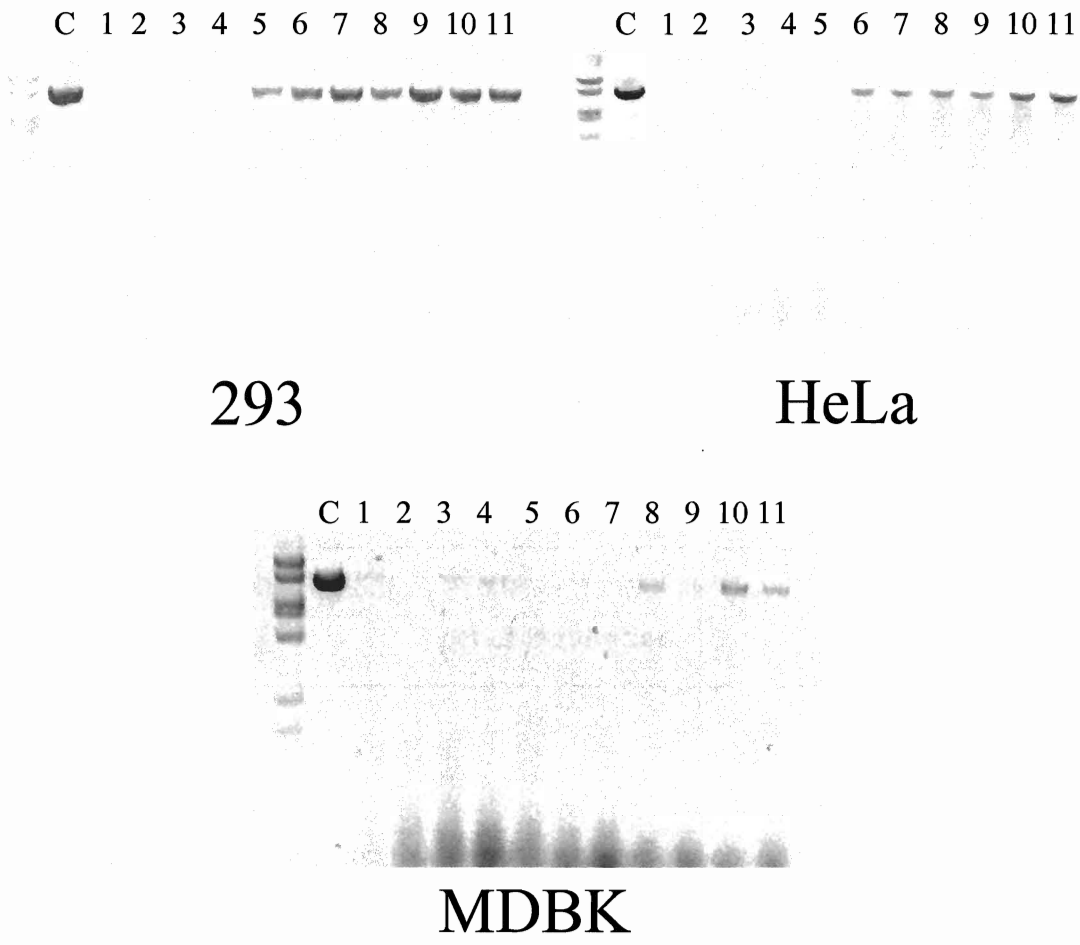


Figure 8: The effects of pH on the DNase activity of various soluble protein extracts. Temperature of incubation was 37°C for 30 minutes (representative gel electrophoresis photo). The lanes are as follows: **C** – Control; **1** – pH 4.0; **2** – pH 4.5; **3** – pH 5.0; **4** – pH 5.5; **5** – pH 6.0; **6** – pH 6.5; **7** – pH 7.0; **8** – pH 7.5; **9** – pH 8.0; **10** – pH 8.5; **11** – pH 9.0; Marker lane – Norgen Biotek FullRanger

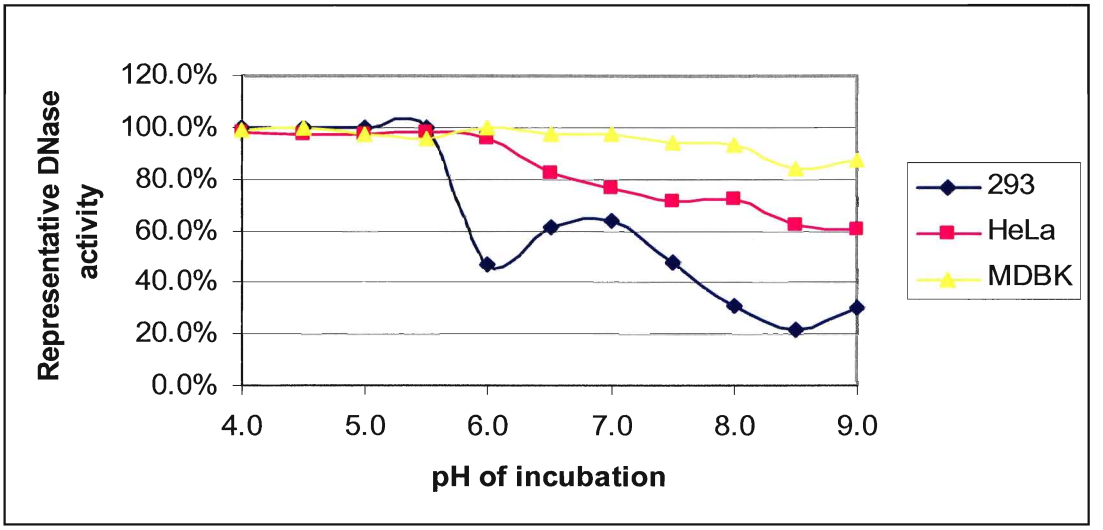


Figure 9: The effects of pH on the DNase activity of various soluble protein extracts using AlphaEaseFC densitometry software. Representative DNase activity (RDA) is obtained by the following formula:

RDA = 100% - (Amount of plasmid in test lane)/(Amount of plasmid in control lane)

The quantification of plasmid DNA is obtained from AlphaEaseFC software using relative densitometry techniques by integral areas. Incubation was performed at 37°C for 30 minutes. Reaction was terminated with a phenol/chloroform extraction. Data is calculated from gel electrophoresis photos of Figure 8.

Visual inspection of Figure 8 yielded the observation that the DNase activity of MDBK cells was extraordinarily high across all pH values from pH 4.0 to 9.0. Because of the faint bands present in the MDBK gel electrophoresis photograph, auto-exposure of the BioRad GelDoc system corrected for the bands by increased exposure time. This resulted in the slight darkening of the entire photo (Figure 8, MDBK), including that of control and marker lanes. Although the gel photograph may appear otherwise, the amount of plasmid DNA used in each experiment during the pH studies were constant. Since all bands were calculated based on internal controls, the calculated values of representative DNase activity and the amount of plasmid remaining were unaffected by the exposure adjustment.

Consistent with the observations obtained from the previous experiment (see section under **DNase activity of various cell lines**), soluble protein extracts of HeLa and HEK-293 demonstrated lower DNase activity than MDBK between the pH values of 5.5 to 9.0. However, all extracts demonstrated the ability to degrade plasmid DNA extensively in the acidic pH values (Figure 9), indicating that a large amount of DNase activity was attributable to the DNase II family of DNases. Based on the observation that DNase II family of DNases was abundant in the protein extracts of all cell lines, including HEK-293 and HeLa, it was reasoned that the likelihood of a member of the DNase II family being responsible for plasmid DNA degradation in relation to transfection efficiency was low, due to the fact that all cell lines had a common denominator, high DNase activity at low pH values. Moreover, Ross *et al.* (1998) reported that DMI-2, a DNase II inhibitor, did not influence transfections using calcium phosphate co-precipitation. Thus, the focus of the knockdown studies was placed on members of the DNase I family of DNases. The argument was that with a more pronounced difference between the DNase activities of the cell lines, the evaluation of the enhancement of transfection efficiency can be assigned to a more measurable difference. However, it cannot unequivocally be determined that DNase II family of DNases did not

contribute to the reduction of transfection efficiency, even in these cell lines. In fact, it has recently been demonstrated that ID2-3, a DNase II inhibitor, resulted in the enhancement of transfection efficiency using PEI and cationic lipoplexes (Sperinde *et al.*, 2001).

Effects of zinc on DNase activity

The inhibitory effects of zinc on DNases have been thoroughly demonstrated in the literature, and its effects encompass both DNase I- and DNase II family of DNases (Laskowski, 1971 and Torriglia *et al.*, 1997). While it was observed that plasmid DNA disappeared over time when incubated in the presence of the soluble protein extracts, contributed presumably by the DNase activity within the soluble protein extract, demonstration of the inhibitory effects of zinc would allow us to attribute the disappearance of the plasmid DNA to DNase activity, rather than DNA binding proteins. Although the phenol-chloroform extraction step, which acted as the termination step of the incubation, made it unlikely that DNA binding protein could have affected the disappearance, a measurable inhibitory effect by zinc would solidify the fact. Using the plasmid digestion assay, zinc chloride was supplemented at concentrations from 0.1μM to 3mM to examine whether zinc could inhibit the disappearance of the plasmid DNA, when incubated with the soluble protein extracts.

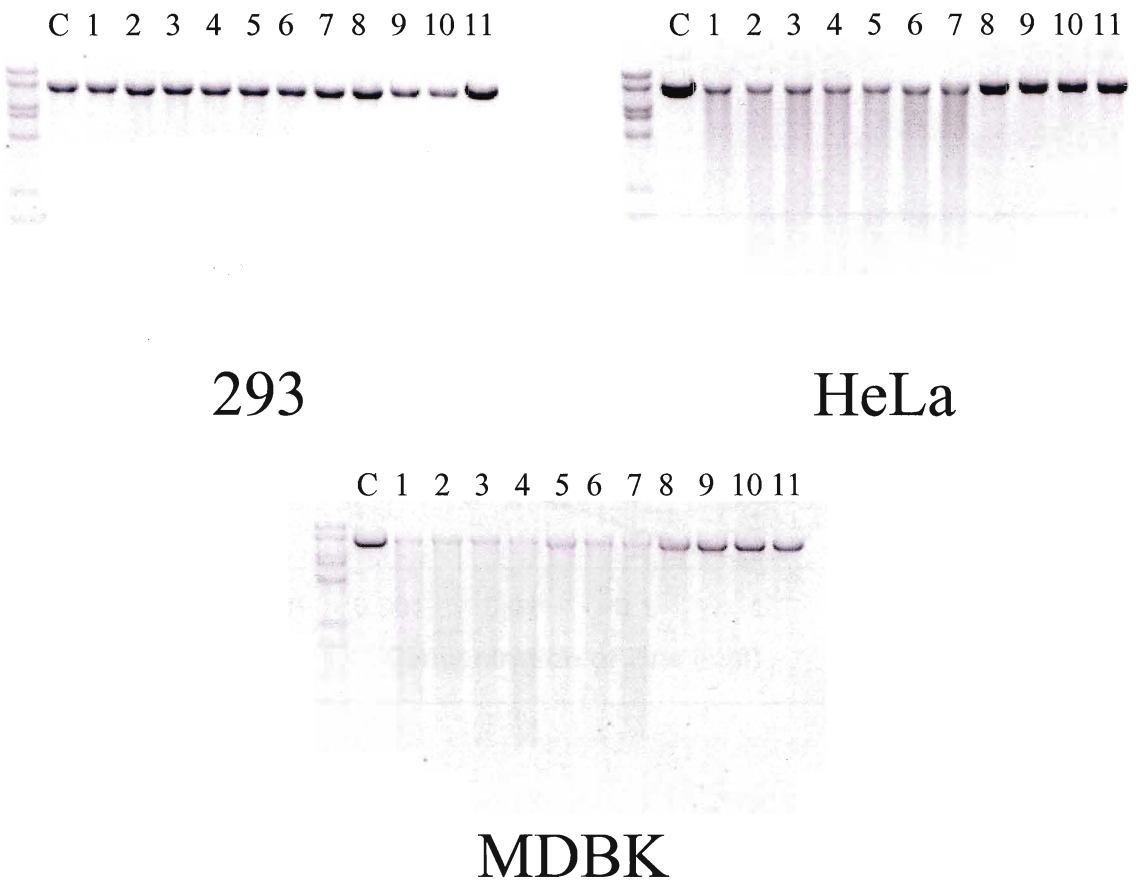


Figure 10: The effects of zinc ions on the DNase activity of various soluble protein extracts. Temperature of incubation was 37°C for 30 minutes (representative gel electrophoresis photo). The lanes are as follows: **C** – Control (no zinc, no soluble protein extracts); **1** – Control 2 (no zinc); **2** – 0.1μM (concentrations of zinc); **3** – 0.5μM; **4** – 1.0μM; **5** – 2.0μM; **6** – 5.0μM; **7** – 10.0μM; **8** – 0.1mM; **9** – 1.0mM; **10** – 2.0mM; **11** – 3.0mM; Marker lane – Norgen Biotek FullRanger

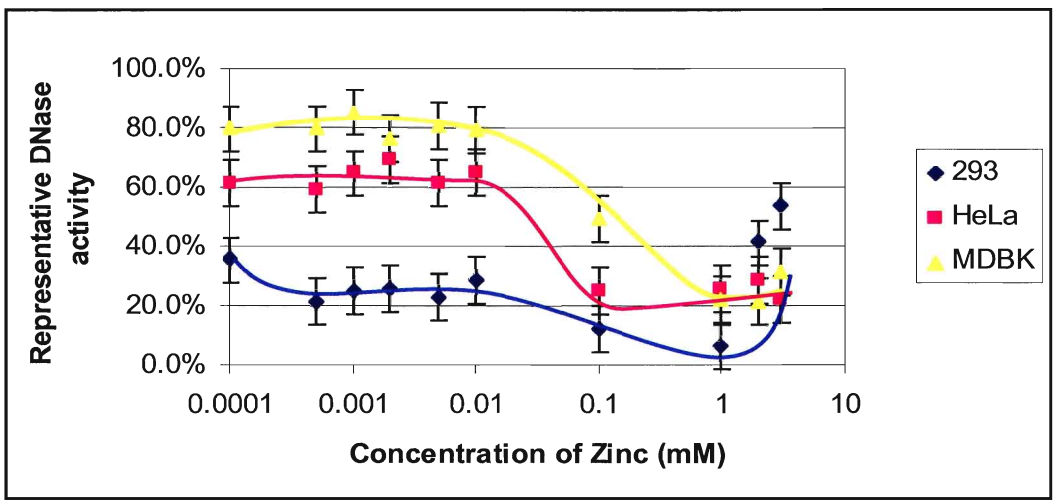


Figure 11: The inhibitory effects of zinc ions on the DNase activity of various soluble protein extracts using AlphaEaseFC densitometry software. Representative DNase activity (RDA) is obtained by the following formula:

RDA = 100% - (Amount of plasmid in test lane)/(Amount of plasmid in control lane)

The quantification of plasmid DNA is obtained from AlphaEaseFC software using relative densitometry techniques by integral areas. Incubation was performed at 37°C for 30 minutes. Reaction was terminated with a phenol/chloroform extraction. Data is generated by averaging the results of 3 trials.

Figure 10 shows that zinc was able to inhibit the DNase activity within the soluble protein extracts across all cell lines in a concentration dependent manner, confirming that the disappearance of DNA during the incubation was due to DNase activity, not DNA-binding proteins. Figure 11 shows that zinc began to show inhibitory effects on DNase activity at concentrations greater than 10μM (0.1μM for HEK-293 extracts, 10μM for HeLa extracts and 10μM for MDBK extracts). At a concentration of 1mM, zinc inhibited nearly all DNase activity, even for the highly active MDBK soluble protein extracts (Figure 9, lanes 9 for all cell extracts).

Since zinc has been documented as an inhibitor of both DNase I- and DNase II families of DNases (Laskowski, 1971 and Torriglia *et al.*, 1997) and has the potential to form complexes with DNA (Kejnovsky and Kypr, 1998), the above results prompted the strategy of using zinc during transfection as a broad-base inhibitor of DNases (see section **The use of zinc in $CaPO_4$ transfection as a means of enhancement**).

An anomaly was observed in both Figures 9 and 10. At zinc concentrations above 1mM, zinc alone caused the degradation of plasmid DNA during incubation, which is a well documented phenomenon in literature (Torriglia et al, 1997).

Effects of aurintricarboxylic acid (ATA) on DNase activity

One of the first reports of the use of ATA, as an inhibitor of nucleases, was published by Hallick *et al.* (1977). As a potent inhibitor of DNase I family of DNases, ATA has not been shown to be an inhibitor of DNase II. If ATA was observed to be an inhibitor of the DNase activity in the soluble protein extracts, it would further strengthen the argument that members of the DNase I family of DNases are primarily responsible for the nuclease activity. While ATA has shown to be discriminatory between the members of DNase I family of

DNases based on inhibitory concentration at 50% (IC_{50}), the use of ATA to distinguish between the various members of DNase I family was not possible because the soluble protein extracts contained all of the members of DNase I family in various proportions. The anticipated effect would be that the overall IC_{50} of the soluble protein extract would lean toward the IC_{50} of its most predominant member, but may lead to erroneous conclusions. Thus, using the same plasmid digestion assay, ATA was supplemented at concentrations of 1μM to 300μM to examine for inhibitory effects on the DNase activity of the various soluble protein extracts.

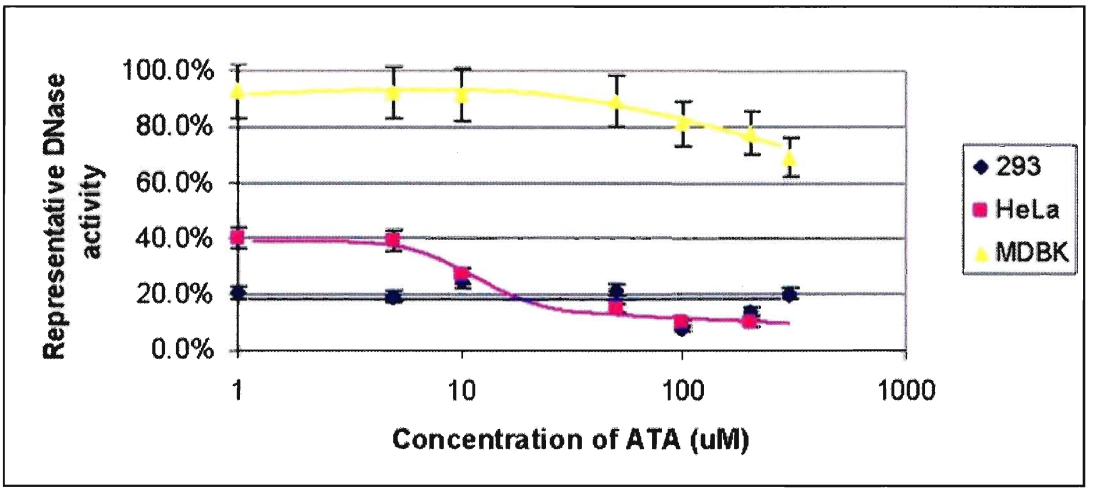


Figure 12: The inhibitory effects of aurintricarboxylic acid (ATA) on the DNase activity of various soluble protein extracts using AlphaEaseFC densitometry software. Representative DNase activity (RDA) is obtained by the following formula:

RDA = 100% - (Amount of plasmid in test lane)/(Amount of plasmid in control lane)

The quantification of plasmid DNA is obtained from AlphaEaseFC software using relative densitometry techniques by integral areas. Incubation was performed at 37°C for 30 minutes. Reaction was terminated with a phenol/chloroform extraction. Data is generated by averaging the results of 3 trials.

As can be seen in Figure 12, the DNase activity of MDBK soluble protein extract was beginning to show signs of inhibition at ATA concentrations above 50μM, which would translate into an IC_{50} value higher than the reported value of 50μM for recombinant DNase I. Similarly, inhibition of DNase activity was also observed for HeLa, albeit at a lower concentration of 5μM ATA. HEK-293 extracts, on the other hand, did not seem to show any significant inhibition. A possible explanation for the lack of inhibition was that HEK-293 extracts exhibited low DNase activity in the first place, and thus, the sensitivity of densitometry studies resulted in differences that were not quantifiable, indicative of the detection limit of this method of analysis. A more likely scenario, however, was that the presence of the DNase I family of DNases was lacking in the soluble protein extracts of HEK-293, which was also consistent with the results based on pH profiles of the DNase activity performed earlier. Thus, the lack of observable inhibiting trend by ATA on HEK-293 soluble protein extract agreed with the previous suggestion that the DNase I family of DNases is responsible for the nuclease activity present in high quantities in MDBK cells but absent in HEK-293 cells.

Overall, the use of ATA in the plasmid digestion assay indicated that the presence of ATA resulted in an observable inhibition of DNase activity in the soluble protein extracts of both MDBK and HeLa cell lines. Thus, further examination between members of the DNase I family of DNases was performed.

On a side note, due to the fact that ATA showed inhibitory effects on the DNase activity of MDBK extracts, the use of ATA as a means of enhancing transfection efficiency was examined. However, the addition of ATA in culture media resulted in the death of MDBK cells, even at concentrations as low as 1μM. Thus, the approach was abandoned.

Effects of G-actin on DNase activity

While evidence seemed to suggest that members of the DNase I family of DNases are active in the soluble protein extracts of HeLa and MDBK cell lines, an attempt to further differentiate which DNase seemed to be most active was conducted. While there are numerous members that belong to the DNase I family of DNases, the only members that have been thoroughly characterized are DNase I and DNase γ (DN1L3). Through literature search, monomeric actin, otherwise known as G-actin, was found to be a specific inhibitor of DNase I and not DNase γ (Blikstad *et al.*, 1978 and Shiokawa and Tanuma, 2001). This allowed for the investigation of whether the ubiquitous DNase I was the active DNase highly expressed in MDBK cells. Again, using the plasmid digestion assay, the inhibitory effects of G-actin on the DNase activity were examined on the soluble protein extracts of all three cell lines.

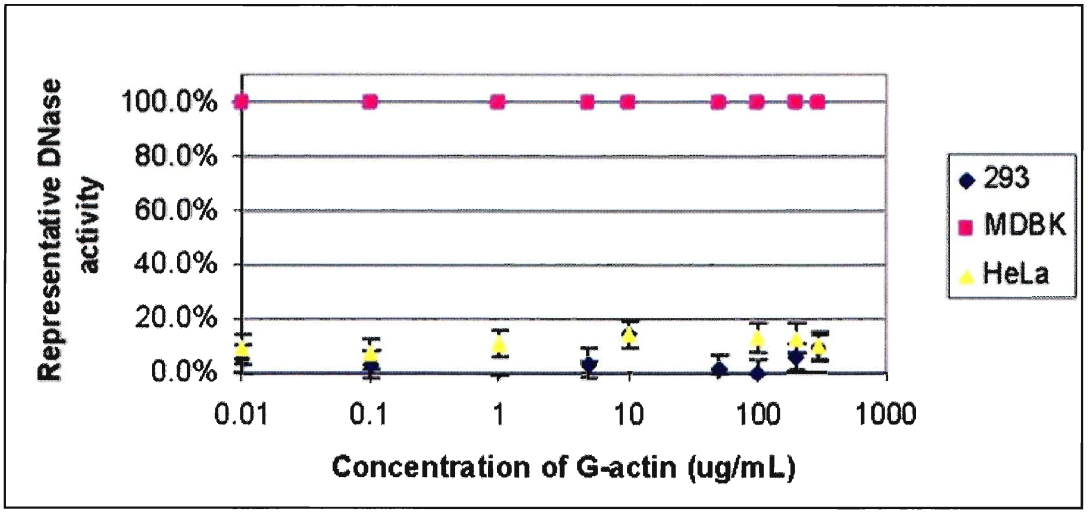


Figure 13: The inhibitory effects of monomeric actin (G-actin) on the DNase activity of various soluble protein extracts using AlphaEaseFC densitometry software. Representative DNase activity (RDA) is obtained by the following formula:

RDA = 100% - (Amount of plasmid in test lane)/(Amount of plasmid in control lane)

The quantification of plasmid DNA is obtained from AlphaEaseFC software using relative densitometry techniques by integral areas. Incubation was performed at 37°C for 30 minutes. Reaction was terminated with a phenol/chloroform extraction. Data is generated by averaging the results of 3 trials.

Figure 13 illustrates that G-actin, at concentrations up to 300μg/mL, did not result in an observable reduction in the DNase activity in the soluble protein extracts from the MDBK cell line. Based on other published reports, the IC_{50} of G-actin on purified recombinant DNase I was estimated at 2μg/mL (Shiokawa and Tanuma, 2001). Thus, the lack of inhibition at 300μg/mL of G-actin suggested that DNase I alone could not account for the nuclease activity observed in MDBK. In fact, it seemed that DNase I activity was conspicuously absent. While it cannot be ruled out that the overlapping activities of DNase I and DNase γ could have skewed the results, such that the total inhibition of DNase I alone was not sufficient to elicit an observable decline in DNase activity because of the presence of high amount of DNase γ. Nonetheless, the inability of G-actin to inhibit DNase activity from the soluble protein extracts from MDBK cells allowed for the assertion that high amounts of DNase γ activity are at least present in MDBK cells. While a participatory role of DNase I in the nuclease activity cannot be excluded, based on the evidence from the inhibition studies, it is concluded that the likely candidate whose inhibition may cause an observable increase in transfection efficiency is DNase γ.

The use of zinc in $CaPO_4$ transfection as a means of enhancement

Since zinc was shown to be a potent inhibitor of DNase activity in the soluble protein extracts (see section **Effects of zinc on DNase activity**) and has been reported to complex with DNA (Kejnovsky and Kpyr, 1998), an approach of utilizing zinc during $CaPO_4$ transfection was devised. It was reasoned that any inhibition of cellular DNases would allow for greater transfected plasmid stability, resulting in the enhancement of transfection. The

approach consisted of: (1) adding zinc during the normal calcium phosphate technique, (2) culturing MDBK cells with supplemental zinc, and (3) using zinc as a substitute for calcium during the transfection procedure.

Using data obtained from Figure 11, DNase activity was shown to be inhibited at concentrations of zinc at 10μM or above. Zinc has been shown to be toxic to cultured MDBK cells and thus the concentration of zinc that can be tolerated was empirically determined. Based on previous optimization studies, the shortest amount of time during which calcium phosphate-DNA co-precipitate was in contact with the cells was 4 hours. Thus, various amounts of zinc (in the form of zinc chloride) from 1μM to 10mM were incubated for 4 hours on MDBK cells to test for toxicity. Medium was replaced after four hours, and cells were visually inspected the next day. At 100μM or above, the toxicity of zinc was apparent as cells began to show more rounded morphology followed by detachment from the culture dish. Further refinement of concentration indicated that at 500μM, all of the MDBK cells detached from the culture dish, and thus, zinc supplement was not practical for use during transfection studies. Additional toxicity of zinc was observed when DNA co-precipitate was added to the culture dishes. Concentrations of zinc below 100μM began showing toxicity, as characterized by the detachment of cells, when DNA-calcium phosphate coprecipitate was added in conjunction with zinc to the cells. Only extremely low concentrations such as 1μM and 10μM did not result in significant cell death (Refer to Table 7).

Table 7: The effects of zinc on MDBK cells.

Conc. of zinc	1μM	10μM	100μM	200μM	300μM	500μM	800μM	1mM	10mM
No DNA	-	-	+	++	++	+++	+++	+++	+++
$CaPO_4$ DNA ppt	-	+	++	+++	+++	+++	+++	+++	+++

- denotes no lifting of cells
+ denotes 0-15% lifting of cells
++ denotes 15%-30% lifting of cells
+++ denotes 90%-100% lifting of cells

The evaluation of transfection efficiency of MDBK cells treated with zinc became difficult mainly because the addition of zinc resulted in cell populations that were far more likely to become detached during the fixing and staining procedure. Thus, the overall survivability of these zinc treated cells was far lower than that of control cultures. Moreover, the small proportion of cells that remained attached did not demonstrate any significant increase in transfection efficiency over control (without zinc), presumably because the amount of zinc (i.e. 1μM and 10μM) was far too low to effectively inhibit DNase activity *in-vivo*.

Because of the high toxicity of zinc, especially in the presence of calcium phosphate co-precipitate, an attempt was made to bypass the difficulty by using zinc in place of calcium for precipitation. By replacing the calcium phosphate with zinc phosphate, higher concentrations of zinc can presumably be used before toxicity becomes an issue because of the synergistic toxicity of calcium and zinc. However, whether zinc demonstrated the same ability to complex with DNA remained to be seen.

Studies by Kejnovsky and Kypr (1998) had suggested that zinc and other metal cations have the ability to cause DNA sedimentation in the presence of phosphate anions. Moreover, zinc chloride was found to be effective in forming insoluble precipitate with DNA

at lower concentrations than that of calcium, which became an encouraging avenue for further exploration. Adapting the centrifugation assay from Kejnovsky and Kypr (1998), zinc chloride was examined as a calcium chloride substitute under transfection conditions. While zinc chloride was found to be an effective agent in DNA complex formation under transfection conditions, the concentration that was needed to cause DNA precipitation had to be optimized to avoid similar toxicity problems. Under normal circumstances, 12.5mM calcium is used in the transfection reagent, which clearly was far too high if zinc was to be used. Further efforts to reduce zinc concentrations while retaining DNA complex formation were undertaken. It was found that at concentrations of 1mM or higher, zinc retained the ability to complex with DNA efficiently (Appendix B7).

While zinc was able to effectively cause DNA to form insoluble precipitate, the concentration needed for complex formation remained far too high to avoid toxicity problems. Using zinc phosphate at 1mM, transfection resulted in significant cell death as attested to by morphology change and detachment from culture dish (Table 7). Thus, there was not an overlapping concentration of zinc that was both non-toxic while retaining the ability to form precipitate with DNA, and the approach was abandoned.

Chapter IV

Cloning of bovine DNase γ and antisense construct

Antisense Inhibition of DNase γ

The use of antisense technology to knockdown or knockout a specific gene has been extensively investigated in *Caenorhabditis elegans*, *Drosophila melanogaster* and various plant species (Kumar and Carmichael, 1998). While conceptually simple, numerous difficulties during its adaptation to mammalian studies have been well documented. These difficulties include the lack of observable antisense effects, the presence of non-antisense effects and the inability to distinguish antisense effects from non-antisense effects (Branch, 1998). Principally, the sequence information of the targeted gene is used to generate a segment of RNA or DNA oligonucleotide, which can contain a variety of modifications to enhance stability, that is complementary to the targeted mRNA. Upon the binding of the antisense oligonucleotide to the sense RNA, double stranded segment of DNA/RNA hybrid is either targeted for degradation by RNase H (in the case of antisense oligodeoxynucleotides) or translation is simply blocked by the occupation of the start codon (Baker and Monia, 1999). Other mechanisms of antisense inhibition include prevention of mRNA transport, splicing and translational arrest (Jansen and Zangemeister-Wittke, 2002). However, antisense RNA is often not an effective means of gene targeting in mammalian cells for a variety of reasons. Transfection of long dsRNA molecules (>30 nt) into most

mammalian cells has been documented to cause nonspecific suppression of gene expression, as opposed to the gene-specific suppression seen in organisms such as plants, protozoa, insects and nematodes. This suppression has been attributed to an antiviral response, which takes place through one of two pathways. In one pathway, long dsRNAs activate a protein kinase, PKR. Activated PKR, in turn phosphorylates and inactivates the translation initiation factor, eIF2a, leading to repression of translation (Manche *et al.*, 1992). In another pathway, long dsRNAs activate RNase L, which leads to nonspecific RNA degradation (Minks *et al.*, 1979). Yet, various groups have experienced success in using long dsRNA for targeted suppression in mammalian cells, indicating that at least some mammalian cell lines have a reduced propensity to elicit this antiviral response (Yang *et al.*, 2001 and Paddison *et al.*, 2002).

Nonetheless, all gene-targeting methods begin with the sequence information of the intended target. Since bovine DNase γ has not been sequenced, the first step of investigation would be cloning of the gene. Using sequence information available from NCBI, homologs of other mammalian species were analyzed using clustal X software freely available from the web site. (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) (Thompson *et al.*, 1997) The species included were *Homo sapiens*, *Rattus norvegicus* and *Mus musculus*.

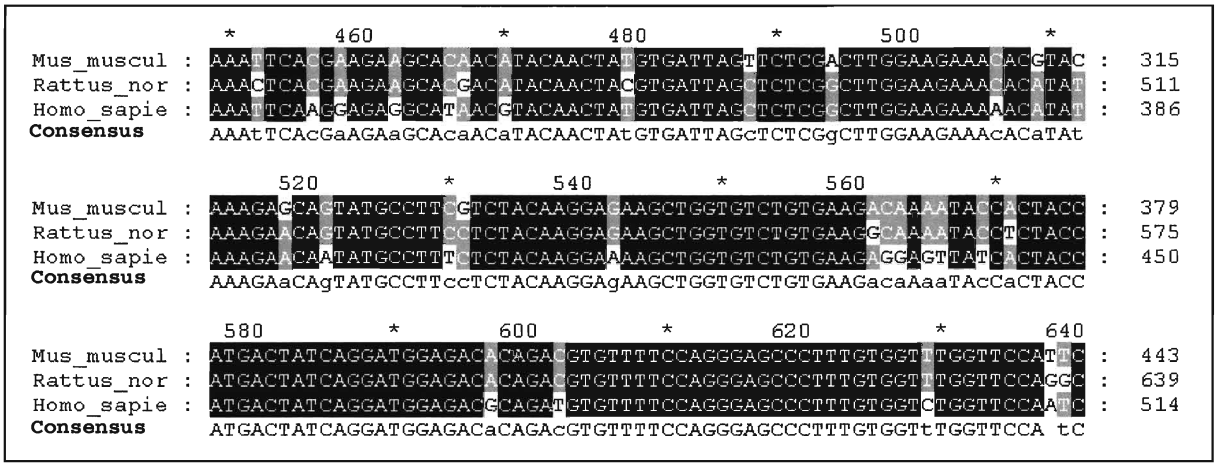


Figure 14: The nucleotide sequence alignment of known DNase γ (*Mus musculus*, *Rattus norvegicus* and *Homo sapiens*) using sequence alignment software Clustal X. Perfect sequence homology between nucleotides 572-597 and 604-628 allow for the generation of PCR primers used in the cloning of bovine DNase γ both in 5' and 3' RACE.

5' RACE

To properly yield primers for PCR, the alignment was analyzed to yield regions of homology that were 25 bases or longer. From the above alignment, there were only two regions of sufficient length, which are presumably the catalytic domains of DNase γ. The homology sequence 5'-CTACCATGACTATCAGGATGGAGAC-3' and 5'-GTGTTTTCCAGGGAGCCCTTTGTGGT-3' which corresponded to nt 572-596 and 604-628, respectively according to the numbering system from the Clustal X alignment, were selected to use as PCR primers for both 5' and 3' RACE.

RNA-ligation mediated Rapid Amplification of cDNA ends (RLM-RACE) was used to clone the DNase γ gene. RNA was isolated using the Norgen RNA-isolation method, which was a silicon carbide based RNA-binding resin, to yield high quality total RNA. For 5' RACE, a RNA-ligase reaction was performed to attach a short segment of RNA of known sequence onto the DNase γ mRNA. Synthesis of the cDNA was done by the reverse transcriptase, Superscript II, as a part of the Reverse Transcription kit (from Invitrogen). Random hexamers were used as primers. The 5' end of the gene was amplified using gene specific primers: 5' GSOP: 5'-GCTGATGGCGATGAATGAACACTG-3' and 5' GSIP: 5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3' by nested PCR. The typical conditions were as described in Material and Methods, with various optimizations. The resulting PCR products were cloned into plasmid vector pUC19 and sequenced based on primers 291F and 552R. The resulting sequence was verified by a BLAST search on the NCBI web site. The sequence of the 5' end of bovine DNase γ is given in Appendix B.

3' RACE

The cloning of the 3' end of bovine DNase γ is similar in approach to the cloning of the 5'end, with a few modifications. After isolation of total RNA, cDNA synthesis was achieved using a modified oligo-nucleotide dT as a primer (additional known sequence is attached to the 5'end of oligo dT). This modification allows for PCR amplification based on the sequences tagged onto the primer. Nested PCR was also performed using 3' GSOP: 5'-3' and 3' GSIP:5'-3' as primers, which were also directed against the nucleotides 572-596 and 604-628 regions, respectively. The resultant PCR fragment was also cloned into plasmid vector pUC19 and sequenced using Amersham's Thermo Sequenase Cy5.5 DNA sequencing kit. The sequencing data was verified by a BLAST search on the NCBI website. Figure 15 shows the homology of the translated 3' EST of DNase γ. The sequence data aligned very well with the homologs of the other mammalian species.

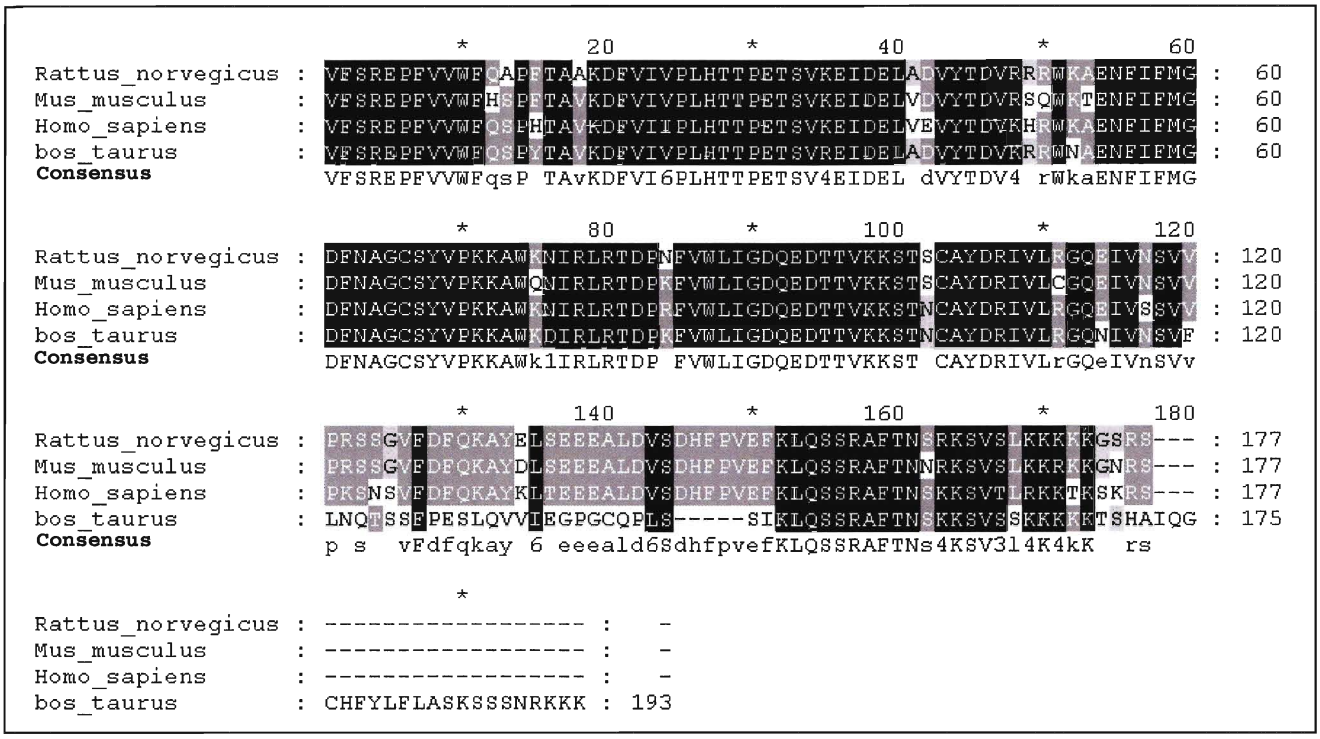


Figure 15: The amino acid sequence alignment of cloned 3' EST of bovine DNase γ with *Mus musculus*, *Rattus norvegicus* and *Homo sapiens* using sequence alignment software Clustal X. For nucleotide sequence alignment, please refer to the appendix.

Construction of antisense expressing vector

To yield a plasmid that constitutively expressed antisense RNA, the 3' EST of DNase γ was subcloned in the opposite orientation under the control of the strong cytomegalovirus (CMV) promoter. Thus, expression of the antisense transcript would yield RNA that is reverse complementary to DNase γ mRNA. Through RNA interference, DNase γ activity should theoretically be reduced by the destruction of DNase γ. For long term selection of the plasmid, the neomycin resistance gene from Tn5, which encodes an aminoglycoside 3'-phosphotransferase, 3' APH II, that confers resistance to the antibiotic G418, was also added to yield antibiotic resistance. Since quantification of DNase γ activity distinguished from other DNases was not possible, selection of neomycin expression is used to infer information about antisense expression. The resultant antisense plasmid, which contains both the antisense cassette and neo^R gene, was named pASDGneoM. Details of the construction and the plasmid map of pASDGneoM are provided in Appendix A.

Chapter V

Antisense disruption of DNase γ and transfection efficiency

The approach to the antisense disruption of DNase γ was three fold: (1) Selection of stable antisense expressing cell lines (2) Co-transfection of antisense construct pASDGneoM and reporter pCMV-β as a transient tool to enhance transfection efficiency and (3) Serial transfection of pASDGneoM followed by pCMV-β, an another means of temporarily disrupting DNase γ. Since it was observed that DNase γ is over expressed in the MDBK cell line and that high DNase activity coincided with low transfection efficiency, any significant amounts of DNase γ disruption should allow for an enhancement of the cytosolic stability of transfected plasmid. Thus, this procedure should translate to an enhancement in transfection efficiency for the MDBK cell line.

Selection of antisense expressing cell lines

The antibiotic, G418, was used as the selective agent for cell lines that have had the plasmid pASDGneoM integrated into the genome. Initially, the stable integration of pASDGneoM was thought to allow cell lines to express continually both neo^R and antisense RNA, which would confer G418 resistance to these cell lines. The evaluation of the effectiveness of antisense RNA by the levels of transfection efficiency enhancement would then follow. Characterization of modified MDBK cell lines that showed enhanced

transfection efficiency would follow to examine the extent of the antisense RNA knockdown. After two weeks of selection at 400μg/mL, 124 G418 resistant MDBK cell lines were isolated. However, upon the transfection of the reporter plasmid pCMV-β, none of the 124 G418 resistant cell lines showed enhanced transfection efficiency. While some of the cell lines transfected at similar levels as that of the control (native MDBK cells), most cell lines became refractory to transfection, not yielding a single transformant per culture dish.

A possible explanation for the lack of observable transfection efficiency enhancement was due to the length of the antisense expressed by pASDGneoM. It has been documented that long dsRNA can cause a non-specific inhibition in translation via either the PKR or 2'-5'-oligoA polymerase pathway (Elbashir *et al.*, 2001). The assumption that a cell line with conferred resistance to G418 would also express the antisense RNA on a long-term basis was erroneous. It is likely that cell lines with both the neo^R gene and the antisense construct integrated would not survive due to the non-specific inhibition of all translation. Thus, it is hypothesized that all 124 G418 resistant cell lines had incorporated only the intact neo^R gene and not the antisense expressing construct, as long dsRNA expression may lead to cell death and thus not viable during the transfection studies. Alternatively, DNase γ served yet another, unknown housekeeping function which is essential to the survival of the cell, whose inhibition would lead to cell death. Yet a third possibility is that the inhibition of DNase γ has little to no effect on transfection efficiency. In any case, based on the current experimental design, elucidation of what led to a lack of observable increase in transfection efficiency was not possible. Since the criterion for characterization was an enhancement in transfection efficiency with pCMV-β, whether antisense RNA was expressed or at what levels were also never fully explored.

An interesting note of neo^R cell lines is that certain cell lines became refractory to transfection. While expression of neo^R may contribute to this effect by affecting central metabolism (Yallop *et al.*, 2003), many commercial cell lines that are G418 resistant (such as HEK293-IQ) transfect in a normal manner. There is a possibility that the site of integration may have caused a non-specific effect in reducing transfection efficiency. However, since the objective was to examine whether the disruption of DNase γ can lead to transfection efficiency enhancement, an alternative approach was devised.

Co-transfection of antisense construct pASDGneoM and reporter pCMV-β

Since it has been reported that long dsRNA causes non-specific inhibition of translation, the use of the antisense construct was combined simultaneously with the reporter plasmid in an attempt to avoid prolonged exposure of long dsRNA. Various combinations of varying amounts of both the antisense construct and reporter were transfected into MDBK cells.

Table 8: Combinations of varying conditions for co-transfection of pASDGneoM and reporter pCMV-β

	Amount of transfected pCMV-β (in μg)	Amount of transfected pASDGneoM (in μg)
pASDGneoM dose response	0.2	1.0
	0.5	1.0
	1.0	1.0
	2.0	1.0
	5.0	1.0
pCMV-β dose response	0.2	2.0
	0.5	2.0
	1.0	2.0
	2.0	2.0
	5.0	2.0

Ultimately, there was no increase in transfection efficiency based on the co-transfection studies (Appendix B5). There are a few reasons as to why co-transfection was less than optimal in terms of transfection enhancement. In the $CaPO_4$ co-precipitation transfection method, there are several key variables that can drastically affect the success of the outcome. One key variable crucial to the formation of fine particles is the concentration of DNA. The concentration of DNA can affect the rate of co-precipitate formation and thus the size of DNA-$CaPO_4$ particles. Ideally, smaller particles are more readily absorbed through the plasma membrane. But with prolonged incubation or high concentration of DNA, larger, less efficient particles are formed (Batard *et al.*, 2001). Based on transfection studies, the optimal amount of DNA to be transfected is 5μg/well, which translates to a concentration of 25μg/mL per transfection volume (Batard *et al.*, 2001 and Figure 16, general trend). From Figure 16, it was observed that deviation from the optimal concentration would adversely affect transfection efficiency (see also Appendix B6). Therefore, it was not surprising that during the co-transfection experiment, additional DNA in the form of pASDGneoM will severely reduce the transfection efficiency because of the formation of

less than optimal particles. Theoretically, a transfection method that is not as concentration sensitive may be more suitable for this study.

Serial transfection of pASDGneoM and pCMV-β

To sidestep the technical difficulties involved in the formation of particles, a series of experiments were designed to examine whether there was another way that the use of the antisense construct would yield enhanced transfection efficiency of reporter pCMV-β. Serial transfections of pASDGneoM were performed. Since the need to use both plasmids at the same time was bypassed, each transfection used the optimal amount of DNA indicated above. As a negative control, plasmid vector pUC19 was used since it does not contain the antisense-expressing construct. First, transfection of the antisense construct pASDGneoM was performed on all MDBK cells. At various time points subsequent to the initial transfection, pCMV-β was then transfected to assess the transfection efficiency, and any enhancement in transfection efficiency would be assessed.

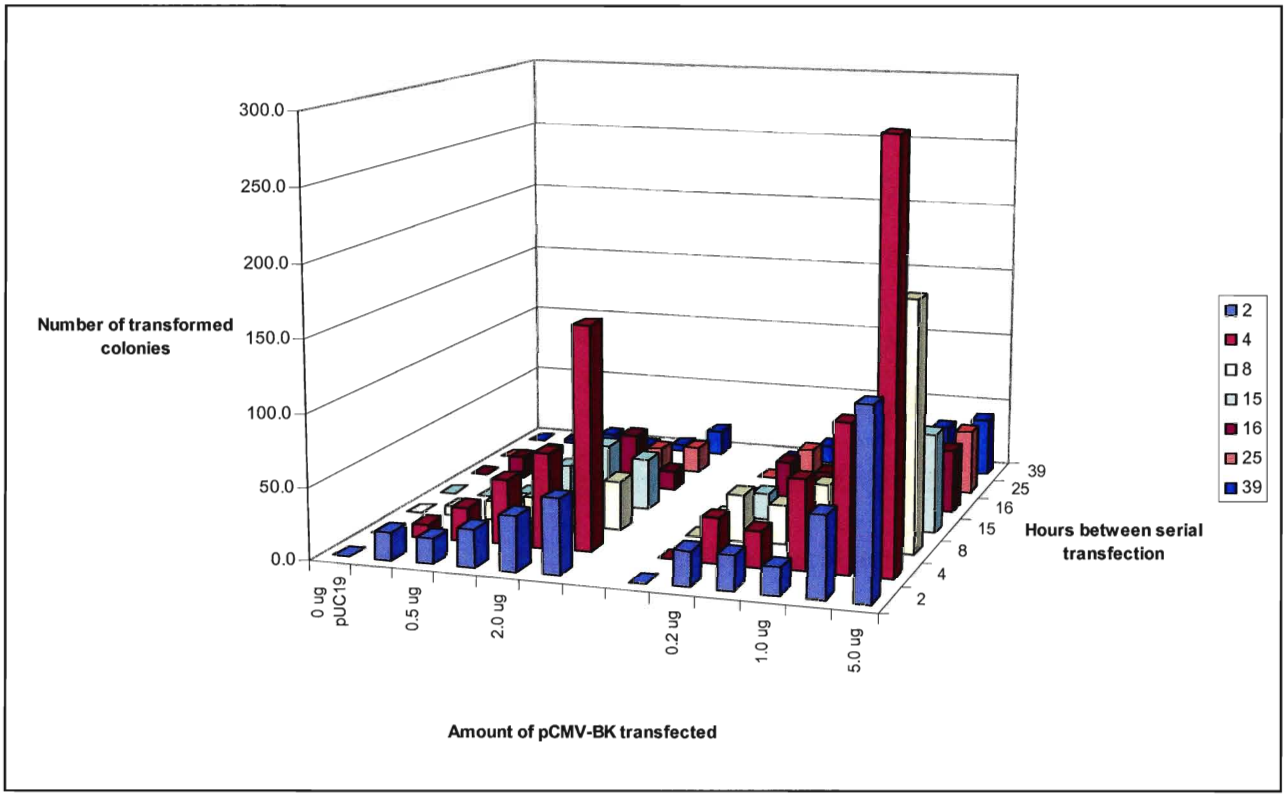


Figure 16: The effects of serial transfection of pASDGneoM and pCMV-β on transfection efficiency in MDBK cells. The cluster of histogram on the left denotes serial transfection results of control plasmid pUC19 and pCMV-β. The cluster of histogram on the right denotes serial transfection results of pASDGneoM and pCMV-β. The amount of time separating the serial transfections is indicated by colors. Increasing amounts of pCMV-β (up to 5.0μg) are used to generate a dose-response curve. The amount of pASDGneoM and pUC19 are fixed at 5.0μg.

In Figure 16, a dose-response relationship is observed between transfection efficiency and amount of reporter plasmid transfected. As the amount of transfected reporter plasmid is increased (up to 5.0μg), an increase in the number of transformed colonies is observed (Appendix B6). The maximum amount of transfected DNA is as outlined by Jordan *et al.* (1996). The cluster histograms on the left shows the data obtained from serial transfection experiments using pUC19 in place of pASDGneoM. Surprisingly, there is also a statistically significant enhancement in transfection efficiency of reporter pCMV-β at 4 hours post-initial transfection as attested by the Dunnette statistical test (at $P<0.05$). The enhanced transfection efficiency eventually decreased back to basal levels after 8 hours.

The cluster histogram on the right is the result of serial transfection experiments using pASDGneoM and reporter pCMV-β. Aside from the general trend of 5μg of reporter yielding the highest number of transformants, there is a significant enhancement of transfection efficiency in the first three time points of serial transfection (2 hours, 4 hours and 8 hours) using pASDGneoM, which was not observed to the same extent in their pUC19 counterpart. Eventually, transfection efficiency decreased back to basal levels (at 15 hours) and the means of the treatment group were no longer significantly different than that of control (single reporter transfection at $P<0.05$).

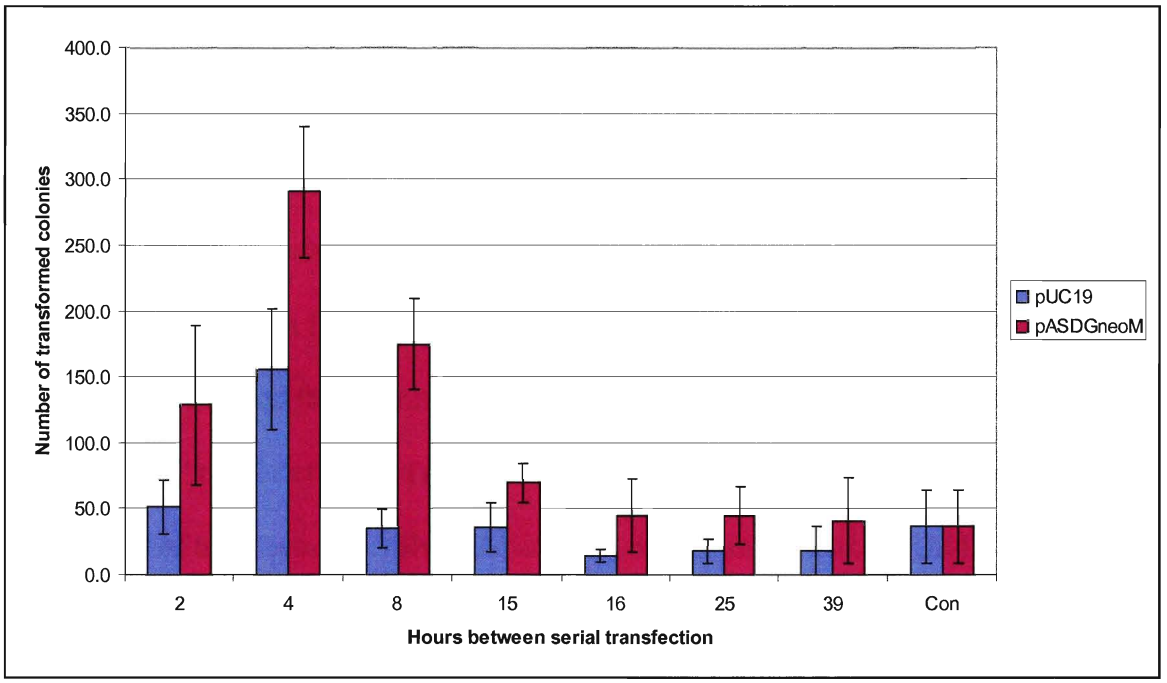


Figure 17: A side by side comparison of the effects of serial transfection of pASDGneoM (or pUC 19) with 5.0μg of reporter pCMV-β on transfection efficiency in MDBK cells. Con indicates the transfection efficiency of a single reporter transfection with 5.0μg of reporter pCMV-β. Data is generated from an average of 3 trials.

Figure 17 provides a side by side comparison of the effects of pUC19 and pASDGneoM in enhancing transfection efficiency. Compared to pUC19, pASDGneoM yielded higher transfection efficiency in all time points. After performing both one-way ANOVA analysis and Dunnette's test, pUC19 yielded a significant variation of the mean at 4 hours while pASDGneoM yield significant variation of the mean at 2 hours, 4 hours and 8 hours.

It was surprising that pUC19 yielded some enhancement in the transfection of the reporter. The most likely mechanism through which pUC19 contributed to the enhancement probably involved the saturation of various nucleases along the delivery pathway, rather than through a "carrier effect". Traditionally, a method of enhancing the calcium phosphate method of transfection involves the addition of high molecular weight DNA known as carrier DNA. While the effects of addition of carrier DNA are highly variable and cell line dependent, it is generally believed that the high molecular weight DNA acted as a carrier to deliver plasmid DNA to the nucleus. Based on this work, it is likely that any extra DNA (in the forms of plasmid DNA) acted as substrates for the DNases present in the cell. Thus, the protective effect of carrier DNA allows for a greater proportion of intact plasmids to escape unscathed through the various stages of degradation. However, in the present studies, the additional carrier DNA had an inhibitory effect on transfection efficiency in the MDBK cell line. In fact, as little as 1μg of additional carrier DNA resulted in over 95% reduction in transfection efficiency of the reporter gene (see Appendix B1). While this effect may have been caused by the suboptimal formation of precipitate due to additional DNA, there is also documented evidence that partially degraded DNA can be cytotoxic, leading to apoptosis (Schiavone *et al.,* 2000).

Since pUC19 theoretically does not contribute to the expression of antisense, the enhancement of transfection efficiency is suspected to be caused primarily by this nuclease

saturation effect. However, sheared genomic DNA should also be able to saturate the DNases but demonstrated an inhibitory effect on transfection efficiency. Since sheared genomic DNA contains more free DNA ends than intact plasmids, this provided a possible explanation why intact plasmid DNA (pUC19) was able to enhance transfection efficiency whereas sheared genomic DNA did not. While both sheared genomic DNA and plasmid DNA can saturate nuclease, sheared genomic DNA also acted as cytotoxic drugs, possibly killing the transfected cell.

Fortuitously, serial transfection of pASDGneoM and reporter resulted in a prolonged enhancement of transfection efficiency. While pASDGneoM can also act to saturate nucleases and thus protect the subsequent reporter plasmid, the nuclease saturation effect alone cannot account for the extra four-fold increase in transfection efficiency over pUC19. A possible explanation is that the plasmid pASDGneoM, once transfected, served to disrupt DNase γ by expressing antisense RNA. Due to the nature of the experimentation, quantification of actual DNase γ activity was not possible.

The disruption of DNase γ, coupled with the nuclease saturation effect, maximally enhanced transfection efficiency at 4 hours up to 8 fold over the single reporter transfection control. Since co-transfection did not yield an enhancement in transfection efficiency (which is akin to serial transfection with no time between transfections), it is suspected that the expression of antisense DNase γ RNA may take a period of time before the DNase environment is altered to the extent where an observable increase in transfection efficiency can occur. Furthermore, it is possible that the short amount of time during which antisense RNA is expressed during serial transfections may have been less prone to triggering the long dsRNA effect seen in the G418 selection experiments.

Based on Figure 17, transfection efficiency slowly declined to control levels after 8 hours. It is likely that the slow decline in transfection efficiency is the result of the intracellular nuclease environment slowly reverting back to the default state. One prediction of this hypothesis would be a faster reversion to basal transfection efficiency levels for the control pUC19 serial transfection than the antisense expression pASDGneoM, which was observed in Figure 16 (15 hours for pASDGneoM vs. 8 hours for pUC19). The reason for the shorter anticipated period of reversion is that the nuclease saturation effect requires continual supply of extra DNA to produce an increase in transfection efficiency, whereas the antisense plasmid would bring about a relatively prolonged change in the nuclease environment.

Additionally, since the density of cells during transfection also plays a role in determining its success, the serial transfection experiments also introduced an extra variable that cannot be controlled. Cells were seeded at a consistent confluence during the first transfection. Upon completing the first transfection, cells continue to grow and divide as expected. If the two serial transfections were spaced over a long period (i.e. more than 8 hours), cells would have grown to suboptimal density for the subsequent reporter plasmid transfection. It seems that at time points of 15 hours and beyond, the density of the MDBK cells may have played a role accounting for a portion of the decline of transfection efficiency over time.

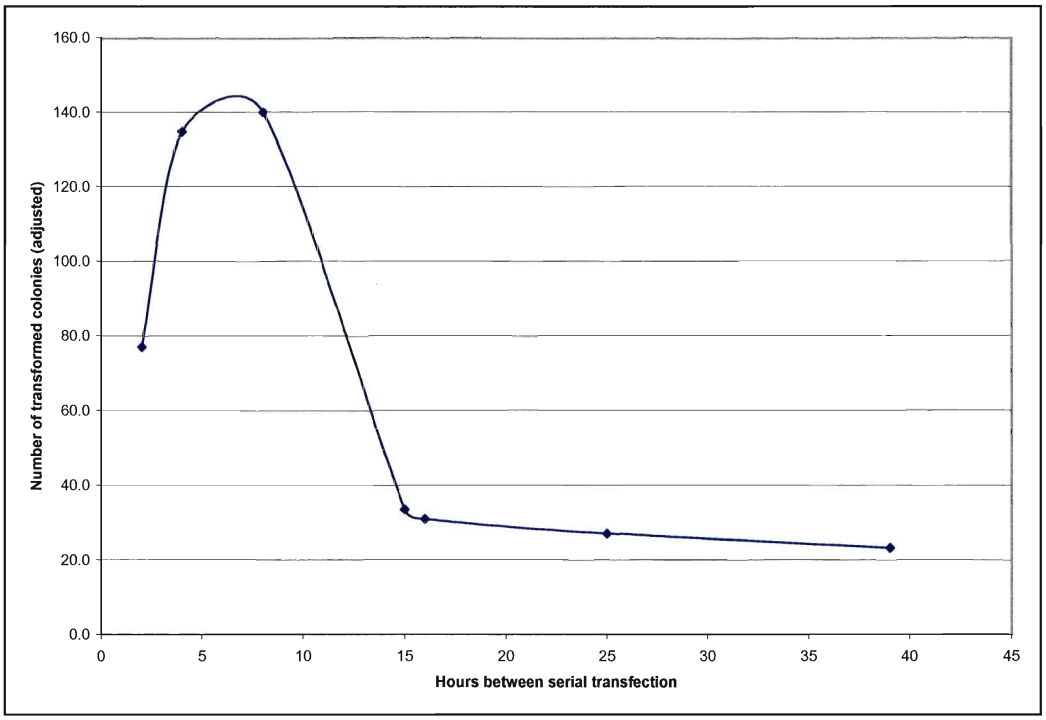


Figure 18: The effects of serial transfection of pASDGneoM and reporter pCMV-β on transfection efficiency in MDBK cells. This figure is compiled by mathematically subtracting the number of transformed colonies generated by serial transfections of pASDGneoM and pCMV-β against that of serial transfection of pUC19 and pCMV-β. The data is plotted against the hours between serial transfection. Raw data was obtained from experiments conducted in Figure 16.

Figure 18 serves to illustrate the time dependence of the success of transfection by subtracting the transfection efficiencies of pASDGneoM by pUC19. While the antisense disruption of DNase γ played a major role in the increase of transfection efficiency, the confines of the experiment led to the observation that cell density could also be a factor, albeit minor, in the success of serial transfection. Together, the enhancement by antisense disruption and the reduction by cell density combined to give a window of opportunity for enhancement of transfection efficiency based on serial transfection of pASDGneoM and pCMV-β. If serial transfections were performed between 4 to 8 hours apart, transfection efficiency can yield up to an 8-fold enhancement over basal levels in transfection efficiency. At 15 hours post-antisense transfection, transfection efficiency reverted back to control levels.

Chapter VI

Construction of an enhanced reporter plasmid, pASDGlacZ

Encouraged by the results of serial transfection, an attempt was made to build the antisense construct and incorporate the reporter into one plasmid to examine whether the presence of the antisense construct will result in enhanced transfection efficiency of the reporter. Using the fragment of the antisense construct pASDGneoM, we inserted the cassette encoding antisense DNase γ under strong CMV promoter into pCMV-β, yielding pASDGlacZ. Both the lacZ gene and DNase γ antisense cassette were cloned in the same orientation (refer to **Appendix A** for construction details). To generate a negative control, a 700 bp non-sense fragment from plasmid vector pNB100 was cloned under the same CMV promoter, yielding pASDGlacZ NC. The plasmid vector pASDGlacZ NC is identical to the plasmid vector pASDGlacZ except for the DNase γ antisense cassette present in pASDGlacZ. The resultant size of the plasmid pASDGlacZ was 8955bp, compared to 7164bp of the parent plasmid pCMV-β.

Transfection of pASDGlacZ, however, did not yield enhanced transfection efficiency over controls (neither the plasmid vector pCMV-β, nor the plasmid vector pASDGlacZ NC). While it was anticipated that the approximately extra 2kb would adversely affect the propensity of the plasmid vector to enter the nucleus, resulting in lowering transfection efficiency, it was surprising to find that the transfection efficiency of pASDGlacZ was not

statistically different than that of the non-sense control plasmid pASDGlacZ NC. One plausible scenario is that the plasmid vector pASDGlacZ does not allow for the requisite amount of time for the expression of antisense DNase γ, at least not enough to change the internal DNase environment to enhance plasmid stability. As a result, the transfection efficiency of plasmid pASDGlacZ would not be enhanced over plasmid pASDGlacZ NC, which was observed in Figure 18. If incorporating the antisense expression cassette does not cause a change in the internal nuclease environment, then the transfection efficiency of plasmid pASDGlacZ would be expected to be lower than that of pCMV-β due to the increase in size, which was observed in Figure 19.

Since there is a requirement to perform the transfection of the antisense construct 4-8 hours prior to transfection of the reporter plasmid, another approach was devised whereby stable cell lines containing antisense expressing constructs integrated into the genome were established. By employing siRNA, certain difficulties encountered using traditional antisense methodology were bypassed (see section **Antisense disruption of DNase γ and transfection efficiency – Selection of antisense expressing cell lines**).

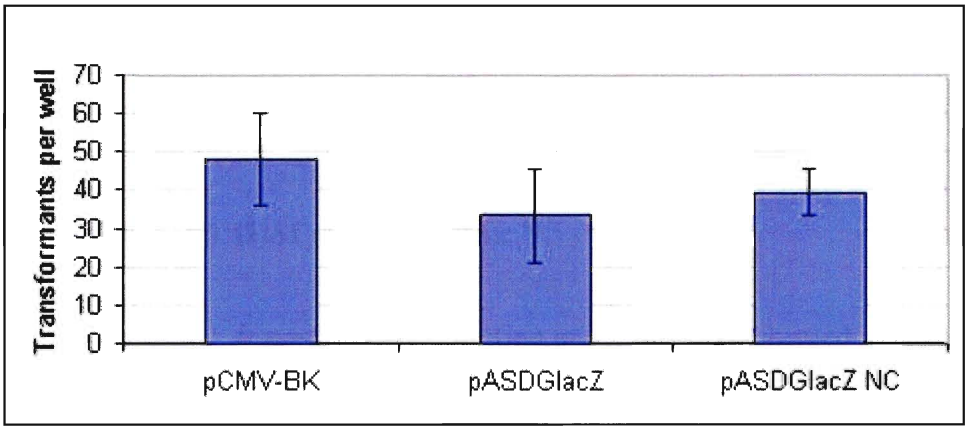


Figure 19: The comparison of transfection efficiency of pASDGlacZ with control plasmid pCMV-β. Transfection is performed using traditional calcium phosphate technique at 25μg/μL per unit volume (5μg/200μL) of cesium chloride banded plasmid DNA. Precipitate formation was left for 10 minutes at room temperature. Transfection occurred for 11 hours and cells were stained 24 hours post transfection. Data presented is obtained by averaging results from 3 trials.

Chapter VII

Use of siRNA to disrupt DNase γ

It was suspected, from the G418 selection experiments performed in chapter IV, that the cell lines that were G418 resistant and refractory to transfection of reporter had somehow integrated only the neo^R gene from pASDGneoM. Since long dsRNA may lead to non-specific inhibition of translation, cells that were expressing the antisense RNA on a continual basis were mostly selected against survival. Shortly after the start of this project, Elbashir *et al.,* (2001) had reported the use of small segments of RNA that can achieve gene disruption by a process known as RNA interference (RNAi), which is a well established technique in plants and *Drosophila melanogaster*. Segments of RNA less than 30 nucleotides that are complementary to the mRNA target can result in a substantial knockdown of gene function. After observing an enhancement in transfection efficiency by serial transfection, attempts to disrupt DNase γ were modified by using the mammalian version of RNAi, known as small interfering RNA (siRNA).

Small interfering RNA refers to gene disruption by short segments of double stranded RNA that are 21 to 25 nucleotides in length. After internalization, these small segments of RNA assemble into endonuclease containing complexes known as RNA-inducing silencing complex (RISC). The RNA segments then guide the RISCs to the target mRNAs, during which the innate ribonuclease activity destroys them. Meanwhile, the potent antiviral

response elicited by long double stranded RNA is completely bypassed (Elbashir *et al.*, 2001).

Using sequencing information obtained from the cloning of the bovine DNase γ gene, the sequence was scanned to match the following criteria:

1. The siRNA sequence must first begin with the dinucleotide AA.
2. The resulting 25 polynucleotide must have a GC content of 30-50%.
3. The target must not contain extensive secondary structure.

Using the sequence information obtained in Chapter IV, 17 sites were obtained to fit criterion 1. Only 3 out of the 17 possible sites had a GC content of 30-50%, termed site 4, site 11 and site 16 (Figure 20 and **Appendix B3**). Since not all suitable siRNA will lead to gene knockdown, all three sites were modified to express hairpin siRNA and cloned into Ambion's pSilencer to enhance the likelihood of success. The plasmid vector pSilencer expresses siRNA by way of a short hairpin, with a segment of palidromic sequences separated by a loop region. It also contains the neo^R gene for selection purposes. The resulting siRNA expressing construct not only allows for selection, but also bypasses the technical difficulties encountered in Chapter V. The sequences from each of the sites were manipulated and the resulting secondary structures were submitted to Mfold for analysis. Moreover, the sequences were submitted to a BLAST search to ensure that the gene knockdown was specific (**appendix B4**). No significant matches other that the intended DNase γ was found.

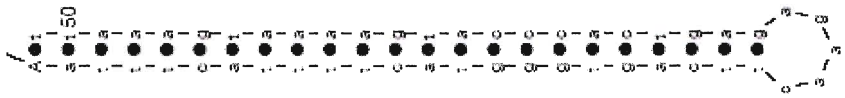
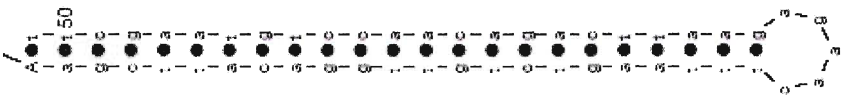
psiRNA S4	AAAAAAATTTCATTTTCATGGGTGACTCTCTTGA AGTCACCCATGAAAATGAAATTGGGAT
Mfold predicted structure	
psiRNA S11	AAAAAAAAGCTTACAGGTTGTCTGAATTCTCTTG AAATTCAGACAACCTGTAAGCTTGGGAT
Mfold predicted structure	
psiRNA S16	AAAAAAAGACCAGTCATGCCTAGATATCTCTTG AATATCTAGGCATGACTGGTCTTGGGAT
Mfold predicted structure	

Figure 20: The sequencing results of the hairpin siRNA-expressing regions of plasmids psiRNA S4, psiRNA S11 and psiRNA S16 (coding strand shown). The Mfold predicted structures of hairpin siRNA expression constructs from plasmids psiRNA S4, psiRNA S11 and psiRNA S14. The sequences in red denote the complementary regions of the hairpin siRNA. The sequences in green denote the loop regions.

After two weeks of G418 selection, 24 established cell lines from each site were screened for enhanced transfection efficiency. Neither site 11 nor site 16 yielded cell lines that reported an enhancement of transfection efficiency that were statistically significant. However, 10 of the 24 G418 resistant cell lines from site 4 yielded enhanced transfection efficiency (Figure 21). Using similar statistical analyses in Chapter V, the transfection efficiencies of only two of the ten cell lines, which were subsequently names B1 and B4, were found to be statistically significant ($P<0.05$).

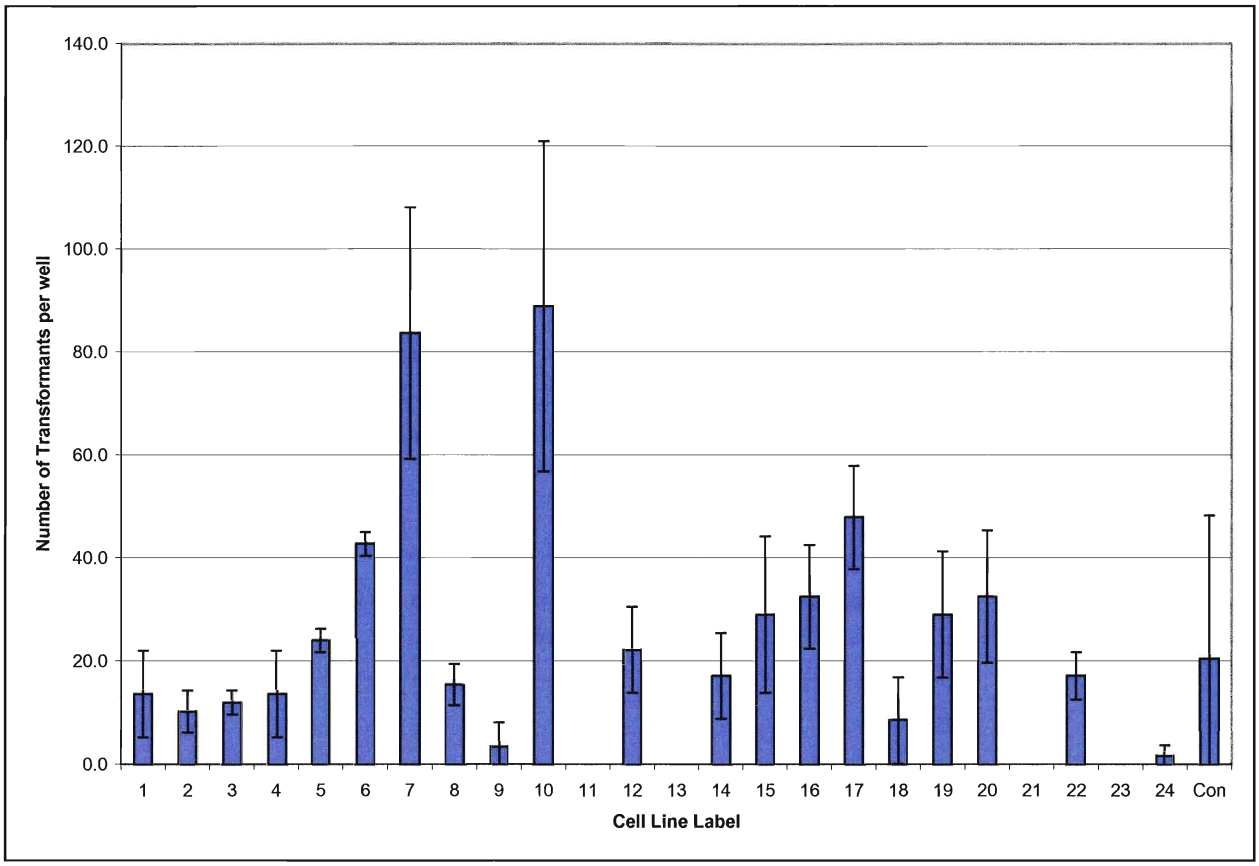
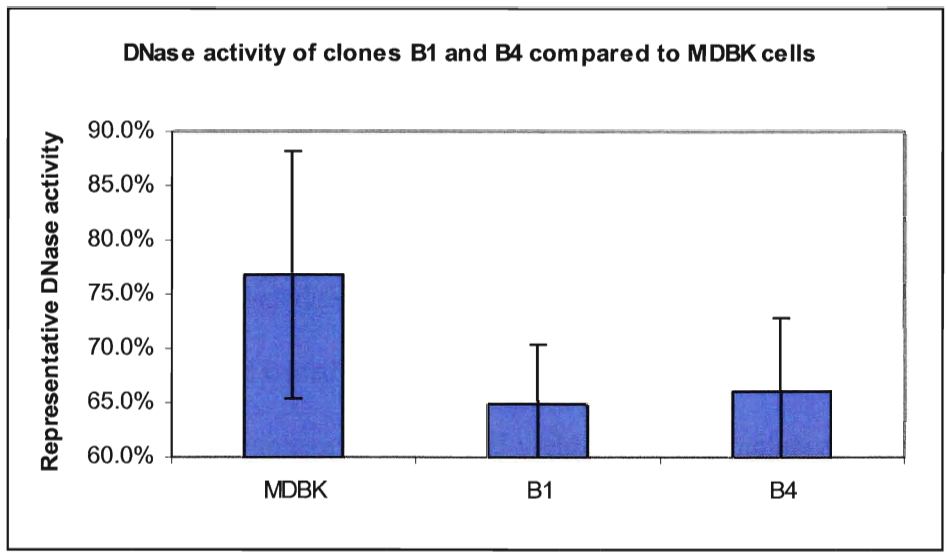


Figure 21: The transfection efficiency of stable psiRNA S4 hairpin expressing MDBK cell lines. Cell lines were transfected with cesium chloride banded plasmid psiRNA S4 and selected with 400μg/mL G418 selection for 2 weeks. Foci that developed during the 2 weeks were isolated with cloning rings and seeded onto a six well plate. Each well is deemed to stem from a homogenous population of one transfected cell and labeled as a cell line. 24 foci were isolated in total and the transfection efficiency using pCMV-β was examined. Transfection is performed as per Materials and Methods. Data presented is an average of 3 trials.



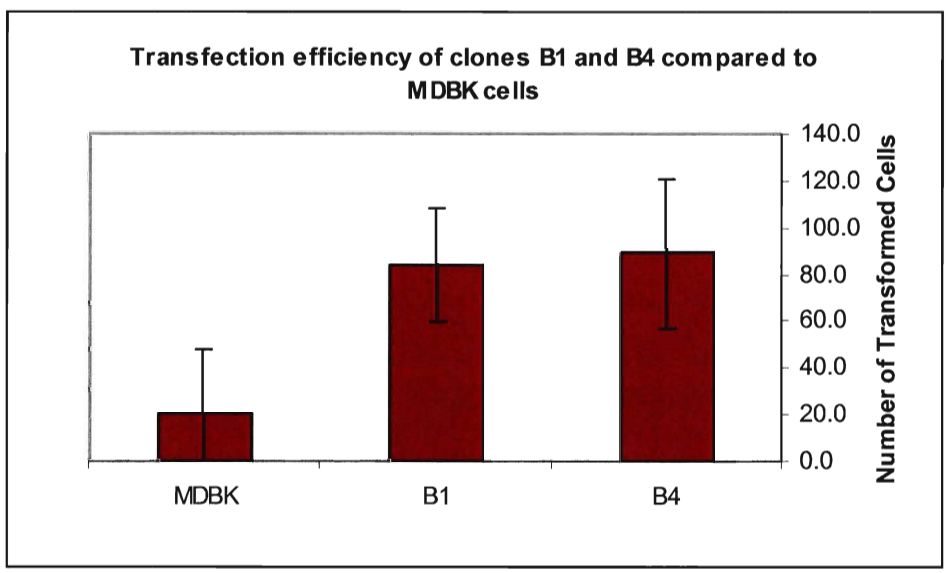


Figure 22: The DNase activity and its corresponding effects on transfection efficiency of psiRNA S4 MDBK cell lines B1 and B4. DNase activity is obtained by isolating soluble protein extract using single detergent solubilization method and performing plasmid digestion assay as per Materials and Methods. Transfection efficiency is obtained by extracting information from Figure 20. Data presented is an average of 3 trials.

The cell lines named B1 and B4 (see Figure 21, cell lines labeled **7** and **10**, respectively) were maintained in low levels of G418 for the stability of the siRNA expressing construct. The transfection efficiency enhancements of both cell lines were approximately 4-fold, coinciding with the results of the serial transfection experiments (B1 - 83.7 and B4 - 88.8 over controls of 20.5 transformants per well). To confirm that the enhancement of transfection efficiency was due to a reduction in DNase activity, DNase activities of the two cell lines were evaluated based on the plasmid digestion assay. Ideally, the inclusion of G-actin into the plasmid digestion assay would yield the most conclusive quantification of DNase γ. However, due to the availability of G-actin, the plasmid digestion assay could only be performed under the most general conditions (pH 7.5 buffer) without the addition of inhibitors. From Figure 22, the soluble protein extracts of cell lines B1 and B4 showed approximately a 10% decrease in overall DNase activity, mainly attributed by the reduction of DNase γ activity. Although the reduction in DNase activity was slight, it led to an effective increase in transfection efficiency, which agrees with the initial hypothesis.

Chapter VIII

Discussion

Since the advent of molecular biology, the need for efficient gene transfer, both in laboratory settings and clinical applications, has driven the development of ever newer DNA complex technology. Historically, non-viral vectors have generally been less efficient in delivering DNA than their virus-derived counterparts. However, safety concerns and immunological difficulties have again refocused effort in the development of non-viral gene transfer.

Ever since the development of calcium phosphate transfection techniques, research efforts have been directed at improving on current DNA complexing agents. The neutralization of the negatively charged DNA backbone and enhanced rate of internalization have both been optimized in a variety of newly developed non-viral transfection agents. However, intrinsic factors of cultured cells can also exert a potent effect on the outcome of transfection studies. For example, certain cell lines, such as MDBK cells, are refractory to transfection regardless of methodology. New DNA complex formation agents have focused on traversing the plasma membrane, which is one of the major barriers of gene transfer. But once internalized, ever-newer generations of transfection agents do not extend the DNA stability against a variety of nucleases present within the cell. Efficient removal of exogenous DNA is a byproduct of DNases that serve several housekeeping functions in

recombination, maintenance of genetic stability and transformation (Baranovskii *et al.*, 2004). In this thesis, the results from soluble protein extract assays revealed a negative correlation between the amount of DNases present and the cell line's transfection efficiency. MDBK cells, notoriously known to be inefficient in gene transfer, also demonstrated a high level of DNase activity. While the amount of nuclease activity present within a cell line is highly variable, whether DNase expression is intrinsic or inducible based on other factors remains a topic of contention. From our laboratory, it was observed that culture conditions of cell lines had a large effect on transfection efficiency. For example, during cell synchronization studies, hydroxyurea and thymidine both resulted in the total inhibition of transfectability in HEK-293 cells. Moreover, cells that were previously overgrown before transfection studies demonstrate a reduction in transfection efficiency against their normal counterparts. Also, as cell lines are passaged, there is a loss of transfectability of HEK-293 cells. It is not known, however, whether age is a contributing factor in the loss of transfectability or whether the lack of selection of adenoviral E1 during cell culture resulted in the inability to transfect. It has been documented, however, that aging animals expressed DNase γ in relation to the apoptotic process; and in the present studies, elevated DNase γ levels correlate negatively with transfection efficiency. Principally, elevated DNase activity in any compartment of the cell can reduce the half-life of exogenous DNA within the cell and thus reduce the amount of successful penetration into the cell nucleus, which is the final barrier for successful transfection.

DNase and transfection efficiency

The association of DNase activity and reduced transfectability in this thesis agreed with the results of Ross *et al.* (1998), who were able to enhance transfection efficiency of the

lung adenocarcinoma cell line H441 by incorporating the DNase II inhibitor, DMI-2. Further studies using peptidal inhibitors of DNase II have also implicated DNase II as a major barrier to transfection. However, according to our studies, the DNase activity that contributes to the inhibition of successful transfection is DNase γ. But it is conceivable that DNase γ may be a major barrier for transfection only in some cell lines. Another interesting observation is that while HEK-293 cells demonstrated an approximate 2 log difference in transfection efficiency over MDBK cells, MDBK cells only demonstrated an approximately 60% greater in overall DNase activity (Figure 7).

Antisense inhibition of DNase γ

Once the bovine DNase γ gene was cloned, the utilization of antisense RNA to inhibit the gene was favored over that of homologous recombination due to the presence of 2 copies of the gene in the cell. To yield homozygous knockouts, 2 separate rounds of selection had to be performed. Traditionally, generation of homozygous knockout mice stemmed from the crossing of two heterozygous knockout mice. Since the exact physiological role of DNase γ was not known, generating homozygous knockouts presented too many difficulties.

Antisense RNA technology has generally been problematic with mammalian cells because of the known potent antiviral response elicited by long dsRNA. However, there has also been documented success with antisense RNA in cultured mammalian cells. Since MDBK has not been documented to be ineffective with antisense RNA, the antisense approach was pursued to examine whether RNA knockdown would yield enhanced transfection efficiency. Since isolation of DNase activity was too laborious to screen in large numbers, bacterial neomycin resistance gene was incorporated into pASDGneoM for

selection. Of the 124 G418 resistant cell lines, none showed elevated transfection efficiency, which was unexpected.

The lack of elevated transfection efficiency in the 124 G418 resistant cell lines may be attributed to dsRNA and internal recombination events. Long-term selection of the selectable marker neomycin resistance required the random integration of the entire pASDGneoM construct. Since it has been documented that long dsRNA results in a general inhibition of translation resulting in cell death, it is likely that either internal rearrangement events excised the antisense expression construct, or during integration, breakage within the antisense construct effectively abolished the expression of antisense RNA. Therefore, cell lines that survived the G418 selection may not have actually carried the antisense expression cassette, and thus no elevation of transfection efficiency was observed. To further make use of the antisense construct pASDGneoM, co-transfection and serial transfection studies were attempted.

Co-transfection was originally the more appealing option because of the ease of performing a single transfection over two serial transfections. However, after the first attempt, a serious pitfall with the co-transfection approach became apparent. Since co-transfection of both the antisense construct and reporter plasmids occurred at the same time, there was not sufficient time for the expression of the antisense construct to significantly alter the internal environment of the cell. Thus, the reporter plasmid experienced similar nuclease activity as that of its native counterpart. Furthermore, the concentration effects of DNA on DNA-calcium phosphate co-precipitate formation yielded additional difficulties to the approach. Therefore, further modification of the co-transfection technique was replaced by the serial transfection method.

Serial transfection, although more laborious, avoided the shortcomings of co-transfection. First of all, since the transfection of the antisense construct and reporter

plasmids occurred in two different phases, each transfection was optimized in terms of DNA concentration. Moreover, transfection of the antisense construct prior to the transfection of the reporter allowed time for the expression of the antisense RNA and gene suppression. When subsequently transfected, the reporter plasmid presumably traveled through a much less hostile environment, leading to more copies of the plasmid surviving their journey to the nucleus. In fact, relative to the single reporter transfection control, the serial transfection of the antisense construct followed by the reporter showed approximately a four to eight fold increase in transfection efficiency under optimal conditions. This enhancement was attributed, as discussed in Chapter V, to the antisense suppression of DNase γ in conjunction with the "nuclease saturation" effect. Unlike the traditional "carrier effect", stemming from the practice of incorporating sheared genomic DNA to enhance the transfection of plasmid DNA, it was concluded that any additional DNA probably acted as a substrate for the various nucleases present within the cell. Also, there was no observable increase in transfection efficiency using sheared genomic DNA as carrier DNA. Worse yet, carrier genomic DNA seemed to only reduce transfection efficiency in MDBK cells, possibly by triggering apoptosis. Using the non-antisense expressing control pUC19 in serial transfection, there was also an unexpected but statistically significant enhancement of transfection efficiency, adding credence to the contribution of the nuclease saturation in enhancing transfection. The antisense construct led not only to an increase in transfection efficiency but also lengthened the amount of time before transfection efficiency reverted back to the basal levels, indicating that a more permanent change in the nuclease environment was brought about via antisense construct pASDGneoM.

Encouraged by the success of serial transfection, an attempt was made to incorporate the antisense expression cassette with the *lacZ* reporter into a single plasmid, pASDGlacZ. This approach was designed to reduce the amount of work needed compared to serial

transfection. Moreover, this approach was advantageous over co-transfection as the concentration of DNA was optimized for a single plasmid carrying both fragments over the simultaneous transfection of 2 plasmids. However, one implicit shortfall of this approach was the requirement of the antisense construct being expressed prior to the introduction of the reporter. As little as 4 hours are needed to sufficiently alter the internal environment for transfection enhancement to occur. Also, as evidenced by soluble protein extract studies, plasmid DNA does not survive more than 90 minutes in native MDBK extract. Thus, it was anticipated that this approach might be problematic because by the time the antisense was sufficiently expressed, the intrinsic nuclease might have degraded the remaining plasmids. This was confirmed by the experimental data, where pASDGlacZ did not show transfection enhancement over its parent plasmid, pCMV-β. While the above reason was most likely attributed to the lack of success of pASDGlacZ, it was also noted that pASDGlacZ was considerably larger than the parent plasmid. Since it has been well documented that transfection efficiency is correlated negatively to the size of the transfection plasmid, this additional hurdle may have exacerbated the issue.

siRNA inhibition of DNase γ

The use of siRNA as a gene knockdown technology presented a unique opportunity, as long-term selection remained the most attractive avenue for studying the involvement of DNase γ in transfection. While bypassing the technical difficulties associated with the traditional antisense approach, an additional advantage of siRNA was that it utilized only small segments of sequence information, meaning that multiple targets could be selected within the gene to find the most effective siRNA sequences. The pSilencer plasmid that was used for siRNA studies was similar in approach to that of the earlier antisense approach. A

neomycin resistance gene for long-term selection was used to identify cell lines with the siRNA expressing cassette randomly integrated into the genome. Of the stable cell lines selected from the three siRNA expressing constructs, site 4 yielded the most promising result. The two G418 resistant cell lines B1 & B4, demonstrated a modest reduction in overall DNase activity while showing a 4-fold increase in transfection efficiency. While it was unexpected to see only a modest reduction in overall DNase activity for both cell lines, the overall improvement in transfection efficiency clearly showed that DNase γ is involved in reducing the stability of transfected DNA in MDBK cells, thereby reducing its propensity for successful transfection to occur.

Enhancement of Transfection efficiency

Generally, transient or stable transfection is a useful tool to study the functions of a particular gene. Transfection methods and cell lines are chosen based on their efficiency and applicability to the problem (for example, MCF-7, a breast cancer cell line, is used to study novel breast cancer therapeutics). The goal in this thesis, on the other hand, was to examine whether a cell line that is generally refractory to transfection, such as MDBK, can be transformed into an efficiently transfectable cell line by manipulating certain genes. In fact, the present thesis demonstrates that by inhibiting DNase γ, transfection efficiency is enhanced by more than 4-fold in MDBK cells.

Apoptosis and Transfection efficiency

But the question remains: Why DNase γ? What is an apoptotic DNase doing inhibiting transfection? As it turns out, many aspects of transfection can have significant

effects on cellular metabolism, resulting in the reduction of transfection efficiency. Plasmid DNA, sheared genomic DNA and calcium phosphate particles can all act as pro-apoptotic triggers (Shiavone *et al.*, 2000), leading to the accumulation of *p53* followed by either cell cycle arrest or apoptosis. In a variety of ways, the accumulation of *p53* around the nucleus leads to the activation of a cascade of signaling events. Based on the state of cellular damage of the cell, these events determine whether *p53* mediates cell cycle arrest, which allows for DNA repair to occur, or apoptosis, via the caspase cascade. In both *p53*-dependent and *p53*-independent pathways, these pro-apoptotic triggers lead to a modulation of DNase γ activity (Higami *et al.,* 2004).

One consequence of this modulation is the degradation of transfected plasmid DNA, reducing the number of plasmids successfully gaining access to the nucleus. Another possibility, under appropriate conditions, is the broad-spectrum activation of apoptosis, leading to the suicide of the transfected cell. Both events pointed to a marked reduction in the probability of successful transfection. As for HEK-293 and HeLa cells, these two cell lines were transformed by viral genomic sequences that seemed to interfere with the function of *p53*. For example, the HEK-293 cell line contains adenovirus type-5 viral genome of the E1 region, and it has been documented that Ad 5 E1B proteins interfere with p-53 mediated cell cycle arrest and apoptosis (Hutton *et al.,* 2000). Moreover, the HeLa cell line, which was transformed by human papilloma virus, conceivably also suffers from certain *p53* defects as it has been shown that HPV E6 viral proteins are potent inhibitors of *p53* (Kessis *et al.*, 1993). The resulting inhibition of *p53* by viral proteins may cause the two cell lines to be less sensitive to DNase γ activation or apoptosis triggered by calcium phosphate, sheared genomic DNA and plasmid DNA. Thus, MDBK may be refractory to transfection not simply because it demonstrates high DNase γ activity. The lack of inhibitors of the apoptotic

machinery presumably leads to enhanced sensitivity of the cell line to pro-apoptotic triggers, which can lead to either the activation of DNase γ or apoptosis, resulting in a cell line that demonstrates low transfection efficiency.

Carrier DNA modulating DNase γ activity

The use of carrier genomic DNA (usually sheared) as a means of enhancing transfection efficiency has been a topic of contention. While some reports have indicated positive results with the use of carrier DNA, some groups have omitted carrier DNA from their transfection protocol citing its inhibitory effects in transfection. In our laboratory, the use of genomic DNA decreased transfection efficiency of MDBK cells with the reporter pCMV-β. Recently, Lepik *et al.* (2003) reported the propensity of sheared genomic DNA to trigger the nuclear accumulation of *p53*, which can lead to cell cycle arrest or apoptosis. It was also shown that exogenous DNA could increase a cell's likelihood to respond to *Fas* ligands and thus trigger apoptosis via the *Fas* apoptotic pathway (de Caravallo Bittencourt *et al.,* 2002). Moreover, strand nicks from a variety of sources led to the activation of poly (ADP-ribose) polymerase-1 (PARP-1), a cellular DNA damage sensor. Together, these observations demonstrated the potential for a cell to recognize transfected sheared genomic (carrier) DNA as cellular DNA damage, possibly activating the apoptosis machinery. Consequently, *p53* accumulation, under the right cellular conditions, could lead to the activation of the caspase cascade, notably the activation of caspase-3. Caspase-3 activation has been shown to modulate DNase1-like 3 (DN1L3), the human DNase γ homolog, via two different mechanisms (Boulares *et al.,* 2002). Caspase-3 has been shown to degrade PARP-1, which has an inhibitory effect on DNase γ activity. Moreover, activation of poly (ADP-

ribose) glycohydrolase further enhances DNase γ activity by removing the poly (ADP-ribose) polymers (Yakovlev *et al.*, 2000). Thus, apoptotic activation of caspase 3 leads to the activation of DNase γ in a *p53* dependent manner.

Plasmid DNA modulating DNase γ activity

Plasmid DNA has also been shown to trigger certain cell cycle signaling pathways when transfected with calcium phosphate. This cellular signaling results in cell cycle arrest, which can be reversed if no other pro-apoptotic stimuli are present. Coincidentally, it has been documented that the intracellular concentration of calcium increases during transfection in CHO cells. Regardless of the source of calcium (whether released from intracellular stores or from the transfection reagent), transfection efficiency seems to be negatively correlated with the intracellular calcium concentration increase. Moreover, it was suggested by Boulares *et al.,* (2002) that the intracellular calcium concentration increase may be a signaling pathway, which eventually results in the activation of DNase γ. Together, the simple act of transfection may be enough to trigger the activation of DNase γ, possibly as a result of the activation of apoptotic machinery. However, the activation of DNase γ can be accomplished in a *p53*-independent manner, as attested by similar results with a $p53^{-/-}$ cell line (Boulares *et al.,* 2002). Furthermore, upon X-ray irradiation, Zhao *et al.* (1999) have also suggested that DNase γ can be triggered in a *p53*-independent manner, as a result of the cell's commitment to die. Thus, the modulation of DNase γ, as a result of exogenous plasmid DNA, can conceivably occur thru a calcium triggered, *p53* independent pathway.

Other factors modulating DNase γ

With several groups recently investigating DNase γ and its role during apoptosis, they have further contributed to the activation mechanism of DNase γ. Tanaka *et al.* (2004) recently demonstrated that aging rats express high levels of DNase γ in their nuclei over their younger counterparts. It is unclear, however, whether the similar age-related phenomenon occurs in immortalized cell lines. Saito *et al.* (2004) also demonstrated the ability of IFN-β to trigger DNase γ in glioblastoma cell lines. However, whether IFN-β results parallel the interferon response triggered by long dsRNA, resulting in the inhibition of transfection of antisense constructs as observed in Chapter V, remains to be seen. Recently, Liu *et al.* (2004) have isolated a protein, Actinin α4, which activates DNase γ in teniposide VM26-induced apoptosis. It is unknown whether Actinin α4 is also an activating signal peptide responsible for DNase γ activity in MDBK cells.

Consequence of DNase γ activation

As a result of DNase γ activation in MDBK cells by transfection, three possible consequences may occur, both leading to the inhibition of transfection. Upon transfection, DNase γ is activated by plasmid DNA, carrier DNA, calcium phosphate or any combination thereof. Upon activation, DNase γ efficiently degrades transfected plasmid DNA, which results in the reduction of successful entry into the nucleus. Furthermore, the ability of DNase γ to translocate to the nucleus also allows for plasmid degradation in nuclear compartments. Together, the end result is the degradation of transfected plasmids and abolishment of observable transgene expression.

If the cell line has accumulated some cellular damage, a more severe response results from transfection. The apoptotic machinery, already partially activated from cellular injury, becomes fully activated upon transfection. Although DNase γ seems to be play a role in the execution phase of apoptosis, the resulting commitment to cell death upon transfection ultimately results in the lack of observable transgene expression due to detachment, regardless of DNase γ activity.

Yet, a third possibility can be the result of apoptosis attributed to DNase γ activity. The innate DNase activity partially degrades transfected plasmid DNA, which is recognized as cellular DNA damage. As the cell cycle arrest machinery is activated, the intracellular calcium concentration increases, caused by the addition of calcium phosphate during transfection, triggers the activation of DNase γ activity. DNase γ then degrades host genomic DNA, which further triggers the DNA damage response and results in the accumulation of more pro-apoptotic triggers. Together, this pushes the cell to commit to apoptosis, resulting in the lack of observable transgene expression.

Summary

Using commonly available cell lines Madine-Darby Bovine Kidney (MDBK), HeLa and Human Embryonic Kidney (HEK-293), a decreasing trend of DNase γ activity was observed based on a plasmid digestion assay. From densitometry studies, as much as a 40% reduction in DNase activity was observed when comparing HEK-293 (least active) to MDBK (most active). Using various biochemical assays, it was determined that DNase γ was expressed more highly in MDBK cell than both HeLa and HEK-293. Upon cloning of bovine DNase γ, antisense constructs via both traditional antisense RNA and siRNA were built. The silencing of bovine DNase γ leads to an increase in transfection efficiency based on traditional calcium phosphate co-precipitation technique; stable clones of siRNA-producing MDBK cell lines (psiRNA-S4) demonstrate a 4-fold increase in transfection efficiency. Furthermore, serial transfection of antisense DNase γ plasmid pASDGneoM and reporter pCMV-β showed a maximum of 8-fold increase in transfection efficiency when spaced 4 hours apart. Together, these results demonstrate that by inhibiting DNase γ, transfection efficiency can be increased by traditional calcium phosphate technique in MDBK cells.

Future Direction

Appendix A. Construction of Plasmids

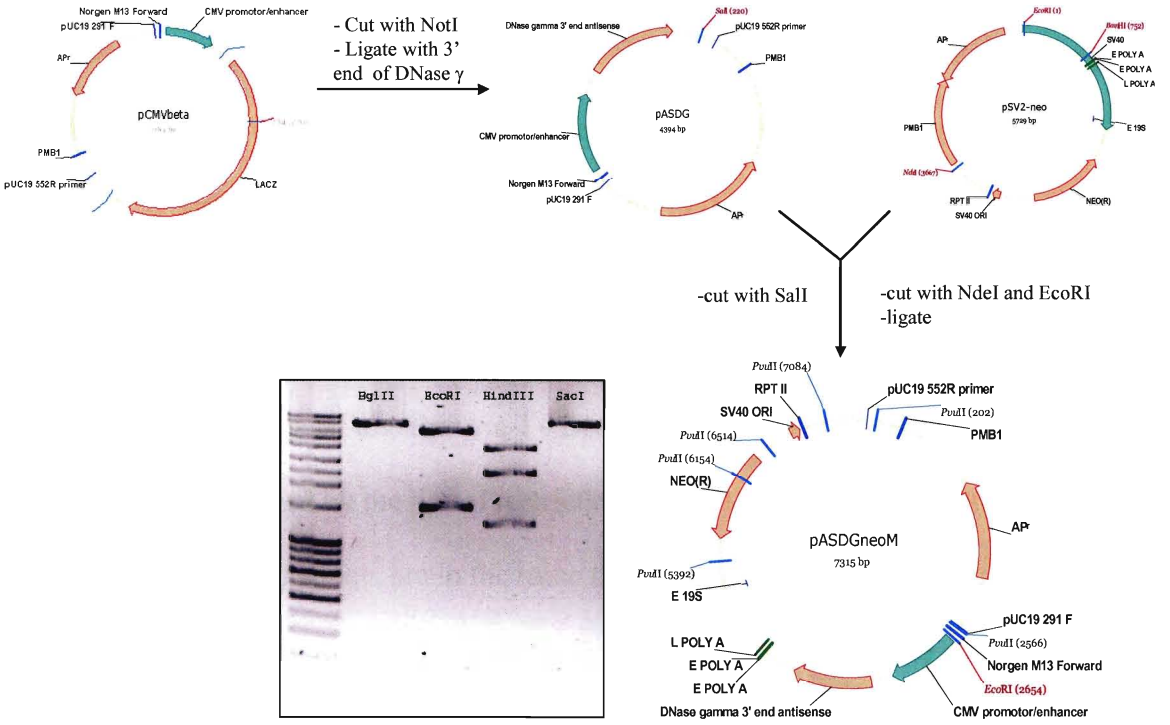
The constructions of the following plasmids are shown below:

1. **pASDGneoM**
2. **pASDGlacZ**
3. **psiRNA S4/psiRNA S11/psiRNA S16**

The assembled sequences of these and other plasmid whose constructions have been described here in the thesis can be obtained from the author or from the supervisor, Dr. Y. Haj-Ahmad.

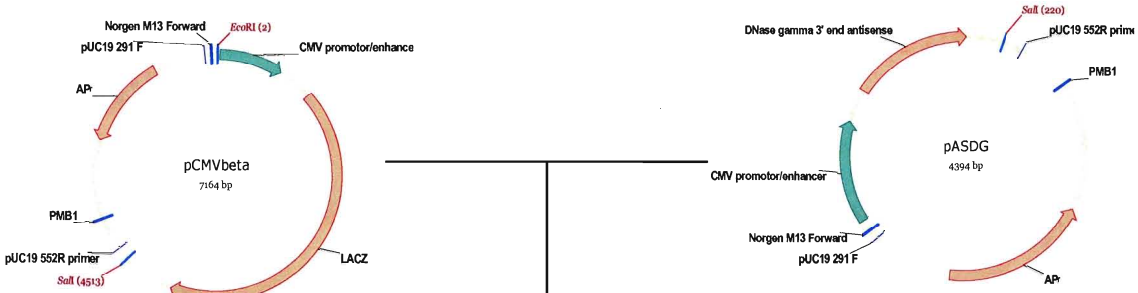
A1. Construction of pASDGneoM

The plasmid vector pCMV-β was cut with restriction enzymes NotI and ClaI, which allowed for the isolation of the fragment containing the pCMV promoter along with the origin of replication and ampicillin resistance gene. The 3' EST of bovine DNase γ was cloned in the opposite orientation to the pCMV promoter to generate the construct pASDG. To add long term selectable marker neo^R, pSV2-neo was cut with NdeI and *EcoRI*, which yielded the neomycin resistance gene under the SV40 promoter/enhancer regions, allowing for expression in mammalian cells. This marker was ligated into the *SalI* restriction enzyme site after rendering the ends of both pieces compatible with *E. coli* polymerase I klenow fragment. The resultant plasmid, named pASDGneoM, contains the antisense bovine DNase γ expressing construct, along with amp^R for selection in *E. coli* and neo^R for selection in mammalian cells.


Norgen M13 Forward
CMV promotor/enhancer
pUC19 291 F
APr
pCMVbeta
PMB1
pUC19 552R primer
LACZ
- Cut with NotI
- Ligate with 3'
end of DNase γ
DNase gamma 3' end antisense
SalI (220)
pUC19 552R primer
PMB1
pASDG
4394 bp
CMV promotor/enhancer
Norgen M13 Forward
pUC19 291 F
AP
EcoRI (1)
BmgBI (752)
SV40
E POLY A
E POLY A
L POLY A
AP
pSV2-neo
5749 bp
PMB1
E 19S
NdeI (3567)
RPT II
SV40 ORI
NEO(R)
-cut with SalI
-cut with NdeI and EcoRI
-ligate
BglII
EcoRI
HindIII
SacI
PvuI (7084)
RPT II
SV40 ORI
PvuI (6514)
PvuI (6154)
NEO(R)
PvuI (5392)
E 19S
pUC19 552R primer
PvuI (202)
PMB1
pASDGneoM
7315 bp
APr
L POLY A
E POLY A
E POLY A
DNase gamma 3' end antisense
pUC19 291 F
PvuI (2566)
Norgen M13 Forward
EcoRI (2654)
CMV promotor/enhancer

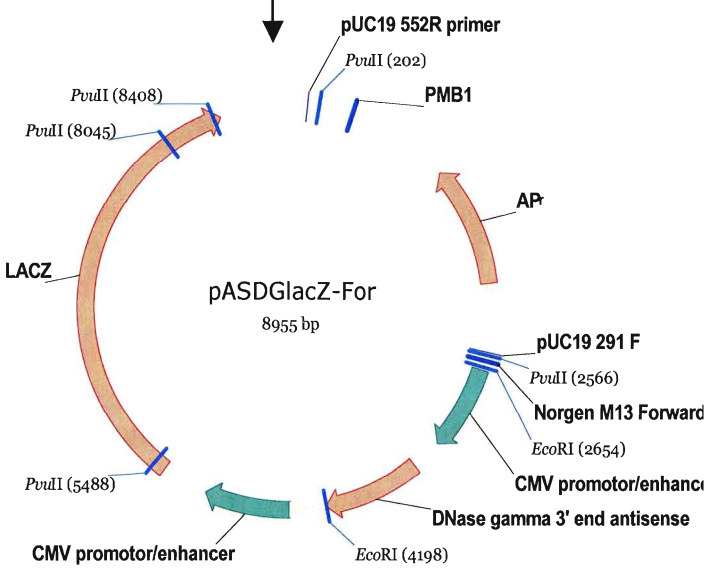
A2. Construction of pASDGlacZ

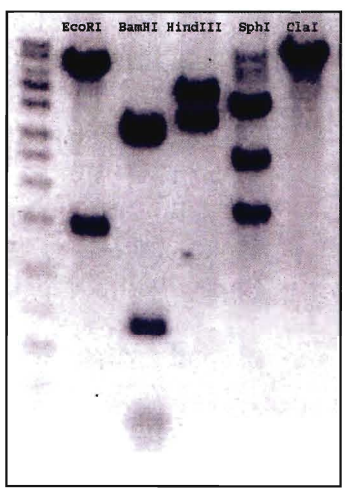
The plasmid vector pCMV-β was cut with restriction enzymes *EcoRI* and *SalI*, which allowed for the isolation of the fragment containing the lacZ gene under the constitutive promoter pCMV-β. The fragment was verified by electrophoresis and purified by gene clean. A klenow reaction was performed to fill in the overhangs generated by restriction enzymes *EcoRI* and *SalI*. The plasmid vector pASDG was linearized by restriction enzyme *SalI*. The overhangs were made blunt by using the klenow fragment. The two blunt ended fragments were ligated together via T4 DNA ligase. The selection for the direction of the inserts was screened by the use of restriction enzyme analysis. The resultant plasmid, in which both the constructs are in the same direction, is named pASDGlacZ-For. It was later renamed pASDGlacZ. The plasmid vector pASDGlacZ has both the antisense DNase γ expressing fragment along with the lacZ gene, both under separate CMV promoters. Also, the plasmid pASDGlacZ has amp^R for selection in *E. coli*.



-cut with *EcoRI* and *SalI*
-gene clean
-ligate

-cut with *SalI*
-klenow
-ligate

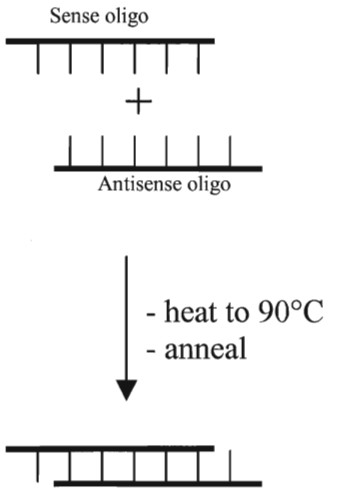


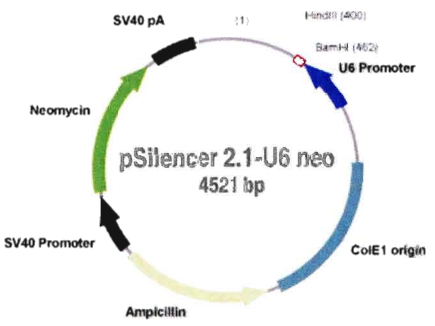


A3. Construction of psiRNA S4, psiRNA S11 and psiRNA S16

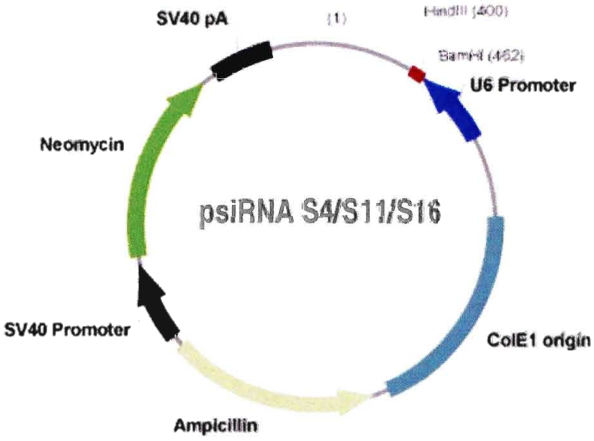
The plasmid vector pSilencer 2.1-U6 neo was obtained from Ambion Incorporated. It contains a neo^R selectable marker under a simian virus 40 (SV40) promoter along with a SV40 polyadenylation signal. The Amp^R selectable marker is used for selection in *E. coli*. The siRNA expression cassette is cloned between the BamHI and *EcoRI* restriction sites, placing it under a mammalian U6 promoter. To generate the various siRNA expressing constructs (psiRNA S4, psiRNA S11 and psiRNA S16), the appropriate sense and antisense oligonucleotides were synthesized. The incorporation of BamHI and *EcoRI* restriction sites to the sense and antisense oligonucleotides, respectively, ensure directionality when cloned into the pSilencer plasmid vector. For example, to generate psiRNA S4, the psiRNA S4 sense oligonucleotides was mixed with the psiRNA S4 antisense oligonucleotides. The two oligonucleotides were heated for denaturation and cooled for annealing. The resultant restriction fragment is cloned into pSilencer cut with BamHI and *EcoRI*. The siRNA hairpin construct also incorporates a PolIII termination signal, with allows for the expression of the siRNA hairpin. Sequencing reactions were performed to verify the accuracy of the three plasmids (psiRNA S4, psiRNA S11 and psiRNA S16) using sequencing protocol outlined in the **Materials and Methods** section.

Oligonucleotide Name	Oligonucleotide Sequence
psiRNA S4 sense oligo	5'-GATCC GTTTCATTTTCATGGGTGAC TTCAAGAGA GTCACCCATGAAAATGAAA TTTTTTGGAAA-3'
psiRNA S4 antisense oligo	5'-AGCTTTTCCAAAAAA TTTCATTTTCATGGGTGAC TCTCTTGAA GTCACCCATGAAAATGAAA CG-3'
psiRNA S11 sense oligo	5'-GATCC GCTTACAGGTTGTCTGAAT TTCAAGAGA ATTCAGACAACCTGTAAGC TTTTTTGGAAA-3'
psiRNA S11 antisense oligo	5'-AGCTTTTCCAAAA AAGCTTACAGGTTGTCTGAAT TCTCTTGAA ATTCAGACAACCTGTAAGC G-3'
psiRNA S16 sense oligo	5'-GATCC GACCAGTCATGCCTAGATA TTCAAGAGA TATCTAGGCATGACTGGTC TTTTTTGGAAA-3'
psiRNA S16 antisense oligo	5'-AGCTTTTCCAAAA AAGACCAGTCATGCCTAGATA TCTCTTGAA TATCTAGGCATGACTGGTC G-3'





-cut with *HindIII* and BamHI
-ligate and transform



Appendix B. Additional Background Data

Additional preliminary data is included here instead of the text of the thesis since these background experiments form the basis of the data included in the thesis. These include optimization of parameters and other information used in the thesis.

1. Effects of Carrier DNA on Transfection Efficiency for MDBK cells

2. Selection of MDBK cells using G418

3. Evaluation of possible siRNA sequences

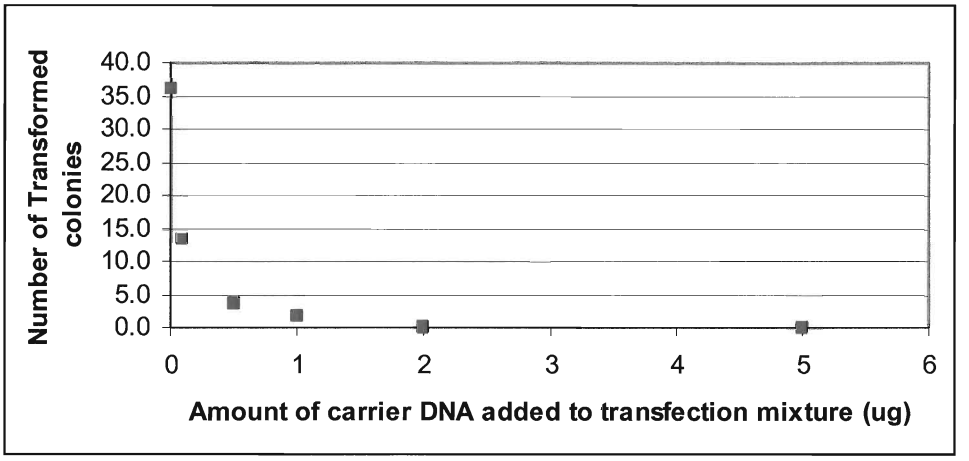
4. BLAST results of siRNA sequences

5. Co-transfection of antisense construct pASDGneoM and reporter pCMV-β

6. Optimization of transfection parameters: Concentration of transfected DNA on transfection efficiency

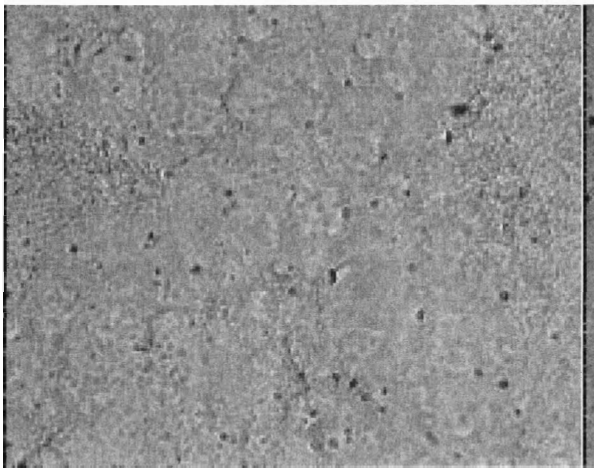
7. Use of Zinc as a Means of Condensing Plasmid DNA for Transfection Purposes

B1. Effects of Carrier DNA on Transfection Efficiency

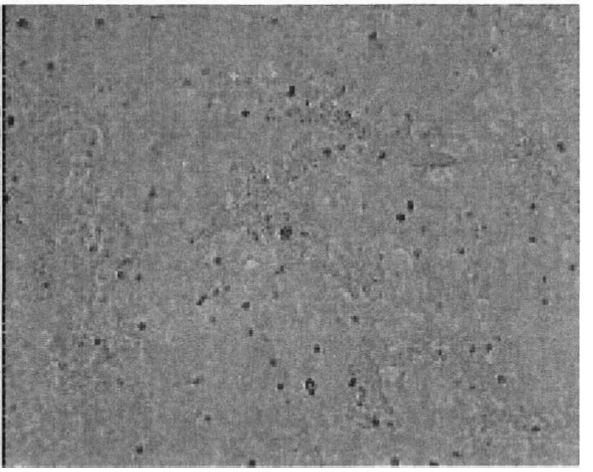


5μg of pCMV-β was transfected along with varying amounts of carrier DNA (sheared salmon-sperm DNA). Transfection is performed as per Materials and Methods. Staining for lacZ activity occurred at 48 hours post-transfection.

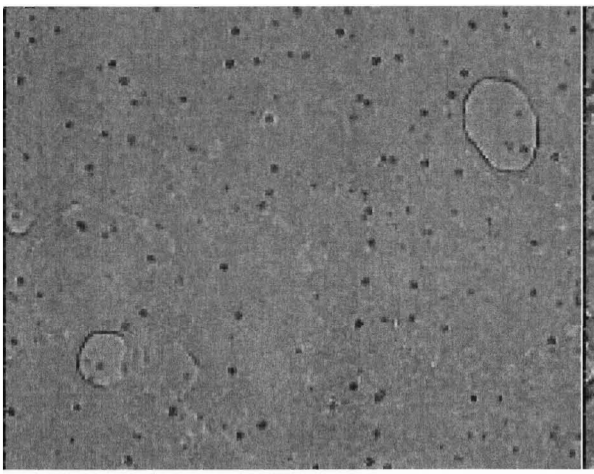
B2. Selection of MDBK cells using G418



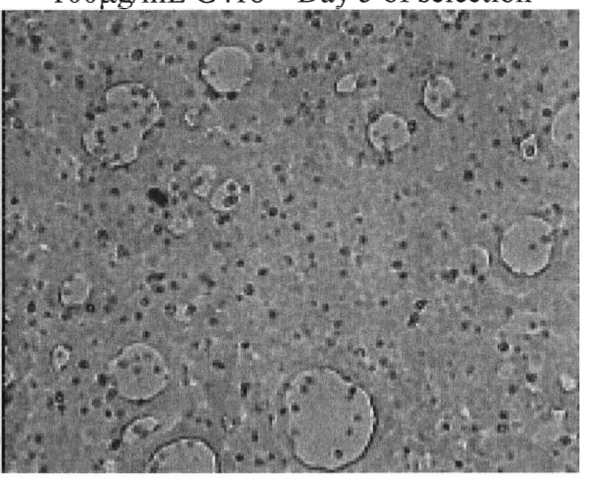
Control – no G418



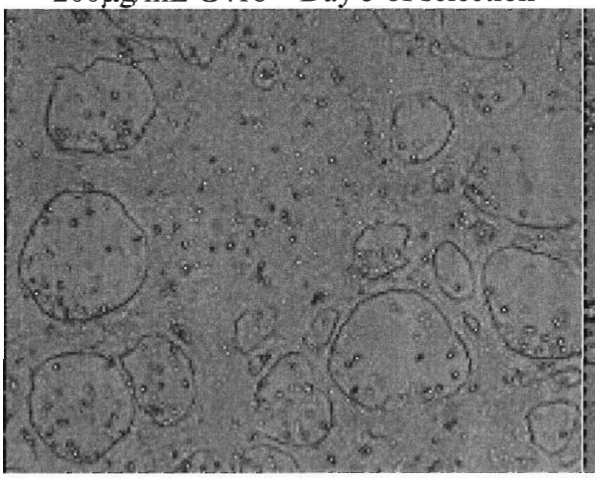
100µg/mL G418 – Day 5 of selection



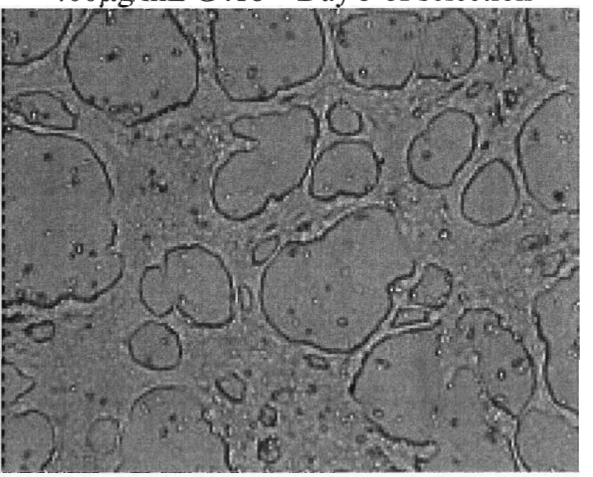
200µg/mL G418 – Day 5 of selection



400µg/mL G418 – Day 5 of selection



800µg/mL G418 – Day 5 of selection



1000µg/mL G418 – Day 5 of selection

Various amounts of G418 were added to the growth media of MDBK cells. Photographs were taken 5 days after selection. For the generation of stable clones (i.e. cell lines conferring resistance to G418 by the expression of neo^R gene), the cells were selected for 2 weeks.

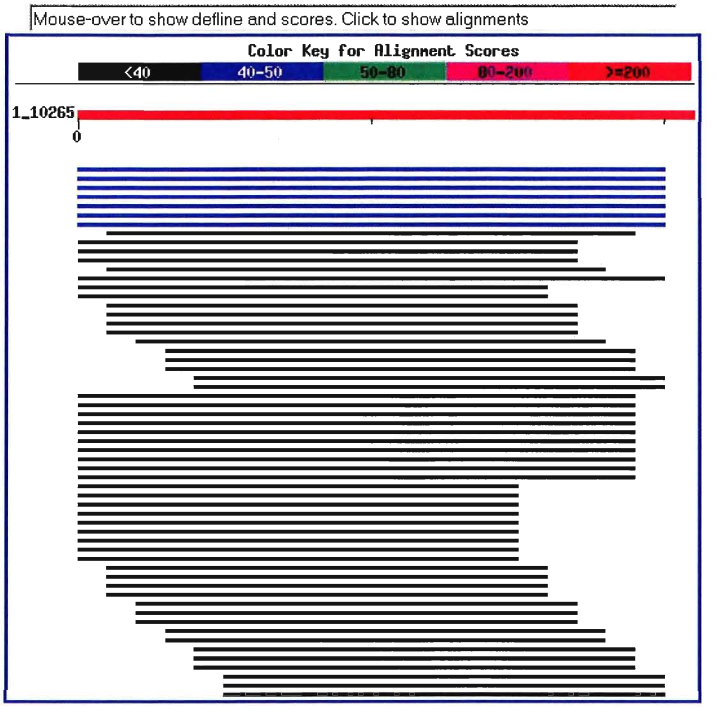
B3. Evaluation of Possible siRNA sequences

	Sequence	Construct	GC content
Site 1	AAGGACTTCGTGATTGTCCCC	AAGGACTTCGTGATTGTCCCC TTCAAGAGA GGGGACAATCACGAAGTCCTT	52.38%
Site 2	AACGTCGCTGGAATGCAGAGA	AACGTCGCTGGAATGCAGAGA TTCAAGAGA TCTCTGCATTCCAGCGACGTT	52.38%
Site 3	AATGCAGAGAATTTCATTTTC	AATGCAGAGAATTTCATTTTC TTCAAGAGA GAAAATGAAATTCTCTGCATT	28.57%
Site 4	AATTTCATTTTCATGGGTGAC	AATTTCATTTTCATGGGTGAC TTCAAGAGA GTCACCCATGAAAATGAAATT	33.33%
Site 5	AATGCTGGCTGCAGCTACGTC		
Site 6	AAGGACATCCGCCTGAGGACG	AAGGACATCCGCCTGAGGACG TTCAAGAGA CGTCCTCAGGCGGATGTCCTT	61.9%
Site 7	AAGTTCGTTTGGCTGATCGGG	AAGTTCGTTTGGCTGATCGGG TTCAAGAGA CCCGATCAGCCAAACGAACTT	52.38%
Site 8	AAGAGGACACCACGGTCAAGA	AAGAGGACACCACGGTCAAGA TTCAAGAGA TCTTGACCGTGGTGTCCTCTT	52.38%
Site 9	AACTGCGCCTATGACAGGATC		
Site 10	AATATTGTCACTCTGTGGTCCT CCATC (BAD)		
Site 11	AAGCTTACAGGTTGTCTGAAT	AAGCTTACAGGTTGTCTGAAT TTCAAGAGA ATTCAGACAACCTGTAAGCTT	38.1%
Site 12	AAGGCCCTGGATGTCAGTGAC	AAGGCCCTGGATGTCAGTGAC TTCAAGAGA GTCACTGACATCCAGGGCCTT	57.14%
Site 13	AACTTCAGTCTTCGAGGGCCT	AACTTCAGTCTTCGAGGGCCT TTCAAGAGA AGGCCCTCGAAGACTGAAGTT	52.38%
Site 14	AACAGCAAAAAATCTGTTTCT	AACAGCAAAAAATCTGTTTCT TTCAAGAGA AGAAACAGATTTTTTGCTGTT	28.57%
Site 15	AATCTGTTTCTTCAAAGAAGA	AATCTGTTTCTTCAAAGAAGA TTCAAGAGA TCTTCTTTGAAGAAACAGATT	28.57%
Site 16	AAGACCAGTCATGCCTAGATA	AAGACCAGTCATGCCTAGATA TTCAAGAGA TATCTAGGCATGACTGGTCTT	42.86%
Site 17	AACTATTTCTTGCCTCTAAAT	AACTATTTCTTGCCTCTAAAT TTCAAGAGA ATTTAGAGGCAAGAAATAGTT	28.57%

Following the criteria stated in Chapter VII, the nucleotide sequence obtain in Chapter IV was screened. 17 possible sites were found beginning with the dinucleotide AA. The sequence is utilized and possible constructs were made by creating a palidromic sequence and inserting a loop region of TTCAAGAGA (green). Of the 17 possible sequences, only 3 sites contained GC content of 30-50%. After cloning the siRNA hairpin into the pSilencer plasmid, sequence verification, Mfold secondary structure analysis and BLAST search were performed (Figure 20 and Appendix B4).

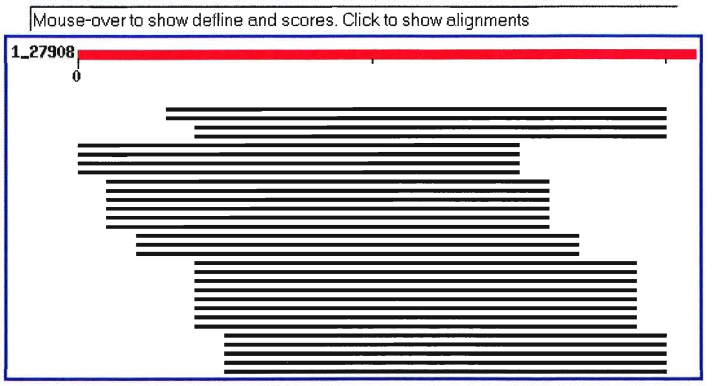
B4. BLAST results of siRNA sequences

Site 4 (refer to B3 for sequence information)



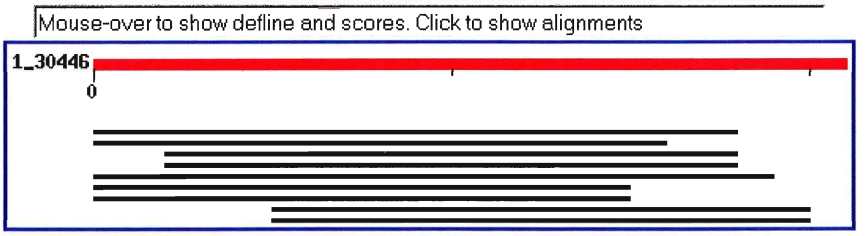
Sequences producing significant alignments:		Score (bits)	E Value	
gi|16198370|gb|BC015831.1|	Homo sapiens deoxyribonuclease I...	42	0.006	L U G
gi|4826697|ref|NM_004944.1|	Homo sapiens deoxyribonuclease ...	42	0.006	L U G
gi|28014555|gb|AC137936.3|	Homo sapiens chromosome 3 clone ...	42	0.006	
gi|19745062|gb|AC098479.2|	Homo sapiens chromosome 3 clone ...	42	0.006	
gi|2905785|gb|AF047354.1|	Homo sapiens liver and spleen DNa...	42	0.006	L U
gi|3236319|gb|U75744.1|HSU75744	Homo sapiens DNase gamma mR...	42	0.006	L U G
gi|1399718|gb|U56814.1|HSU56814	Human DNase1-Like III prote...	42	0.006	L U G

Site 11 (refer to B3 for sequence information)



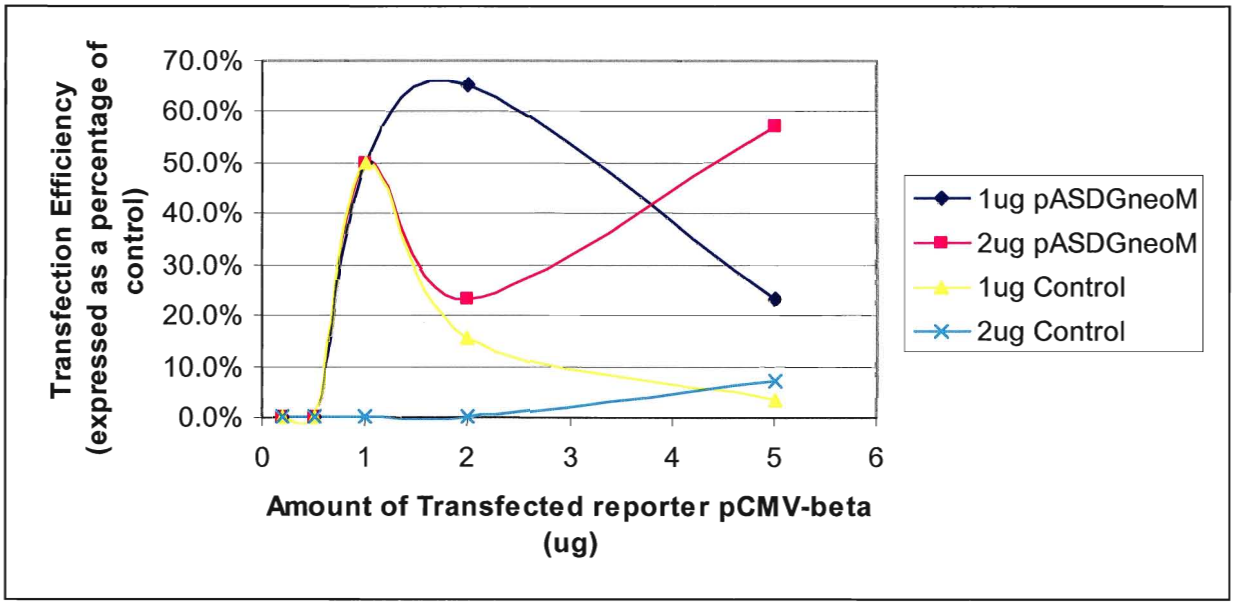
Sequences producing significant alignments:		Score (bits)	E Value
gi|37693683|gb|AC104914.9|	Mus musculus chromosome 9, clone...	36	0.37
gi|9971632|dbj|AP002527.1|	Escherichia coli plasmid R721 ge...	36	0.37
gi|16501141|emb|AL445287.16|	Human DNA sequence from clone ...	34	1.4
gi|20152340|emb|AL390122.16|	Human DNA sequence from clone ...	34	1.4

Site 16 (refer to B3 for sequence information)



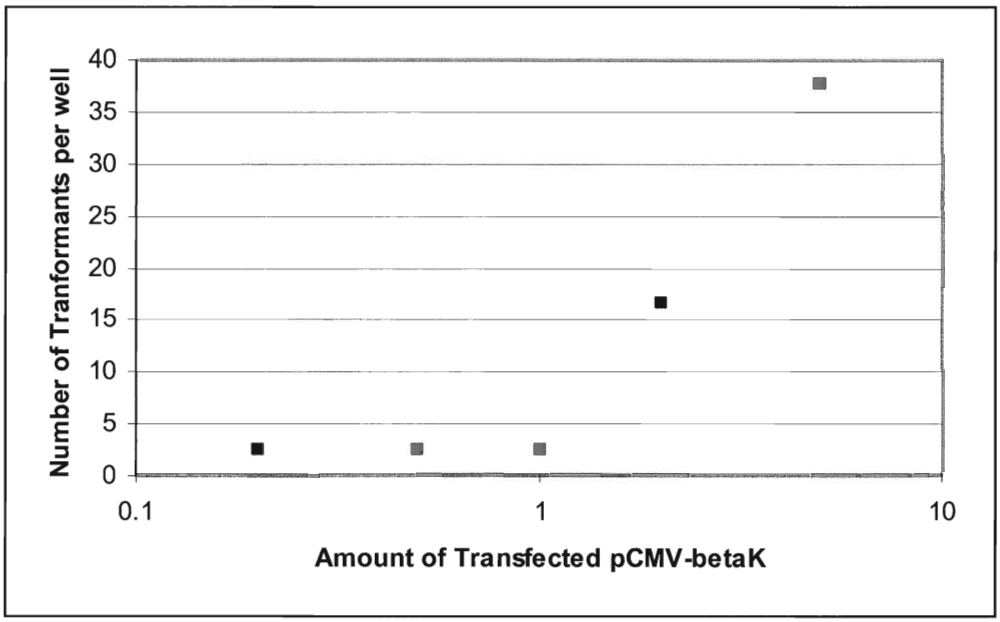
Sequences producing significant alignments:		Score (bits)	E Value
gi|20068630|emb|AL645783.13|	Mouse DNA sequence from clone ...	38	0.093
gi|19774306|gb|AC112918.3|	Homo sapiens X BAC RP11-370L12 (...	34	1.4
gi|17223145|gb|AC097263.6|	Homo sapiens X BAC RP11-298C3 (R...	34	1.4
gi|34221791|emb|AL670024.7|	Mouse DNA sequence from clone R...	34	1.4
gi|10047940|gb|AC011290.3|	Homo sapiens BAC clone RP11-64I2...	32	5.7
gi|34581725|gb|AC116406.12|	Mus musculus chromosome 3, clon...	32	5.7
gi|29788800|gb|AC090950.3|	Homo sapiens chromosome 3 clone ...	32	5.7
gi|20340433|gb|AC117430.3|	Homo sapiens 3 BAC RP11-119D18 (...	32	5.7
gi|12001757|emb|AL390894.2|CNS06C7W	Human chromosome 14 DNA...	32	5.7

B5. Co-transfection of antisense construct pASDGneoM and reporter pCMV-β



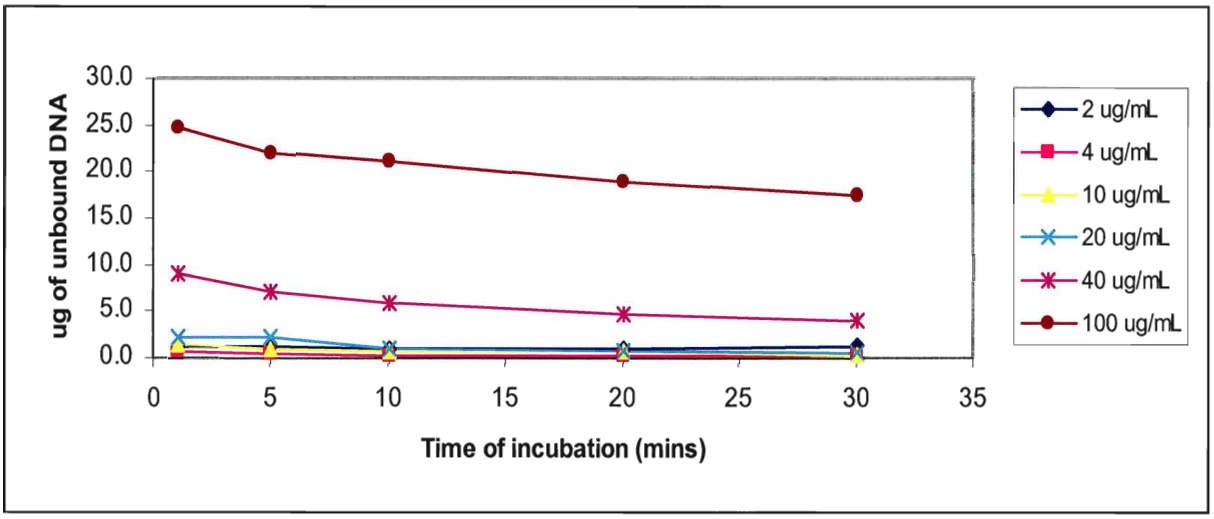
Various amounts of pCMV-β were co-transfected with varying amounts of pASDGneoM (antisense construct) or pUC19 (control). Transfection is performed as per Materials and Methods. Staining for lacZ activity occurred at 48 hours post-transfection. None of the methods resulted in a significant increase over standards (no co-transfected plasmid) as attested by the less than 100% transfection efficiency.

B6. Optimization of transfection parameters: Concentration of transfected DNA on transfection efficiency

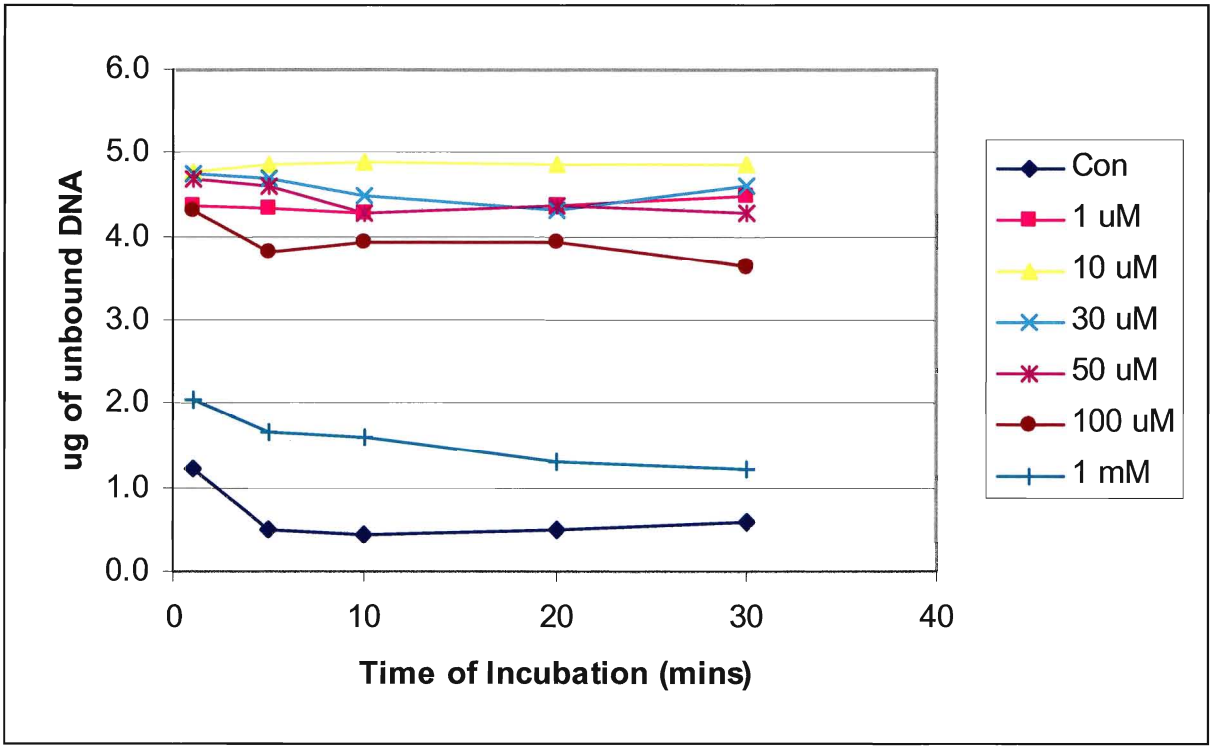


Various amounts of pCMV-β were transfected using traditional calcium phosphate technique. Transfection is performed as per Materials and Methods. Staining for lacZ activity occurred at 48 hours post-transfection.

B7. Use of Zinc as a Means of Condensing Plasmid DNA for Transfection Purposes



Various amounts of plasmid DNA was complexed with 3mM $ZnPO_4$. Amount of unbound DNA was determined by incubating the DNA-$ZnPO_4$ mixture at room temperature and centrifuged at 14,000g for 1 minute. The OD_{260} of the supernatant is used to quantify unbound DNA in solution. At 3mM zinc, concentrations of DNA up to 20μg/mL can be efficiently complexed. Longer incubation period leads to the sequestering of more DNA from solution. However, the nature of the precipitate (i.e. coarse vs. fine) is not quantifiable by this method.



Due to the cytotoxic effects of zinc on MDBK cells, lesser amounts of zinc was tested for efficient complex formation with DNA. 5μg of plasmid DNA was incubated at room temperature with various amounts of $ZnPO_4$. Unlike 3mM $ZnPO_4$, DNA is not efficiently complexed at concentrations less than 1mM. According to Table 7, concentrations of zinc greater than 10μM results in cellular toxicity. Thus, the toxicity of zinc made it impossible as a transfection reagent despite the fact that it complexes DNA efficient than $CaPO_4$. Control denotes 12.5mM $CaCl_2$.

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